

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09385 A2

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number: PCT/US00/20571

(22) International Filing Date: 28 July 2000 (28.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/364,320 29 July 1999 (29.07.1999) US

(71) Applicant: EPOCH PHARMACEUTICALS, INC.
[US/US]; Suite 110, 12277 134th Court N.E., Redmond,
WA 98052 (US).

(72) Inventors: LUKHTANOV, Eugeny, Alexander; 817
205th Street SE, Bothell, WA 98012 (US). PODYMINO-
GIN, Mikhail, A.; 4950 N.E. 193rd Street, Lake Forest
Park, CA 98155 (US). HEDGPETH, Joel; 33 Robinhood
Drive, San Francisco, CA 94127 (US).

(74) Agents: KLEIN, Howard, J. et al.; Klein & Szekeres,
LLP, Suite 700, 4199 Campus Drive, Irvine, CA 92612
(US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— Without international search report and to be republished
upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: ATTACHMENT OF OLIGONUCLEOTIDES TO SOLID SUPPORTS THROUGH SCHIFF BASE TYPE LINKAGES
FOR CAPTURE AND DETECTION OF NUCLEIC ACIDS

(57) Abstract: Derivatized oligonucleotides (ODNs) are coupled to a solid support in improved yield resulting in a high density of coupled oligonucleotide per surface unit of the support, through a Schiff base type bond formed between an NH₂ group attached either to the solid support or to the ODN and an aromatic aldehyde attached to the other of the solid support and the ODN. The preferred solid support-ODN conjugate is formed between semicarbazide groups attached to a glass surface and an aromatic aldehyde attached at either 3', or 5' end of an ODN or to an intermediate nucleotide of the ODN.

WO 01/09385 A2

Document BH
Cited in IDS for 10010381-1
Serial No. Unassigned
filed Herewith

1 ATTACHMENT OF OLIGONUCLEOTIDES TO SOLID SUPPORTS
2 THROUGH SCHIFF BASE TYPE LINKAGES FOR CAPTURE AND
3 DETECTION OF NUCLEIC ACIDS

4 Field of Invention

5 The present invention generally relates to the chemistry of the
6 attachment of oligonucleotides to solid supports. More particularly the present
7 invention relates to linking oligonucleotides to solid supports through a Schiff
8 base type covalent linkage for capture and detection of single- and double
9 stranded DNA and RNA targets.

10 Background of the Invention

11 The detection and quantification of very small quantities of nucleic
12 acids plays an important role in the biological, forensic and medical sciences.
13 Typically nucleic acids in samples are detected by hybridization to a
14 complementary oligonucleotide containing more than 8 contiguous
15 nucleotides. To provide a signal proportional to the target-oligonucleotide
16 hybrid, typically either the target or the capturing oligonucleotide contains a
17 signal generating label, such as a radioactive-, fluorescent-,
18 chemiluminescent- moiety or an enzyme (such as horseradish peroxidase) that
19 through its catalytic activity yields a detectable product. The prior art is well
20 developed in this regard and numerous methods are available for the detection
21 and quantification of signal in the nucleic acid field.

22 Following the hybridization of the capturing and labeled
23 oligonucleotide to the target nucleic acid it is necessary to separate the signal
24 generating duplex from unreacted target and labeled oligonucleotide. This can
25 usually be accomplished because either the target, or more typically the
26 capturing oligonucleotide has been immobilized on a solid support thereby
27 allowing the isolation of the hybrid free from contaminating molecules. In a
28 "sandwich assay" variation, an oligonucleotide is immobilized to a solid

1 support and is used to capture a target. The captured target is detected by
2 hybridization with a second labeled oligonucleotide, that has a different
3 sequence than the capturing oligonucleotide.

4 Numerous types of solid supports suitable for immobilizing
5 oligonucleotides are known in the art. These include nylon, nitrocellulose,
6 activated agarose, diazotized cellulose, latex particles, plastic, polystyrene,
7 glass and polymer coated surfaces. These solid supports are used in many
8 formats such as membranes, microtiter plates, beads, probes, dipsticks etc. A
9 wide variety of chemical procedures are known to covalently link
10 oligonucleotides directly or through a linker to these solid supports. Of
11 particular interest as background to the present invention is the use of glass
12 and nylon surfaces in the preparation of DNA microarrays which have been
13 described in recent years (*Ramsay*, Nat. Biotechnol., 16: 40-4 (1998)). The
14 journal Nature Genetics has published a special supplement describing the
15 utility and limitations of microarrays (Nat.Genet., 21(1): 1-60 (1999)).

16 Typically the use of any solid support requires the presence of a
17 nucleophilic group to react with an oligonucleotide that must contain a
18 "reactive group" capable of reacting with the nucleophilic group.
19 Alternatively, a "reactive group" is present or is introduced into the solid
20 support to react with a nucleophile present in or attached to the
21 oligonucleotide. Suitable nucleophilic groups or moieties include hydroxyl,
22 sulfhydryl, amino and activated carboxyl groups, while the groups capable of
23 reacting with these and other nucleophiles (reactive groups) include
24 dichlorotriazinyl, alkylepoxy, maleimido, bromoacetyl groups and others.
25 Chemical procedures to introduce the nucleophilic or the reactive groups on to
26 solid support are known in the art, they include procedures to activate nylon
27 (US 5,514,785), glass (*Rodgers et al.*, Anal. Biochem., 23-30 (1999)), agarose
28 (*Highsmith et al.*, J., Biotechniques 12: 418-23 (1992) and polystyrene (*Gosh*

1 *et al.*, Nuc. Acid Res., 15: 5353-5372 (1987)). Dependent on the presence of
2 either a reactive or nucleophilic groups on the solid support and
3 oligonucleotide, coupling can either be performed directly or with bifunctional
4 reagents. Bifunctional and coupling reagents are well known in the art and
5 many are available from commercial sources.

6 Of special interest as background to the present invention is the
7 procedure described by *Kremsky et al.* (Nuc. Acid Res., 15: 2891-2909 (1987))
8 for the preparation of a 16-mer oligonucleotide containing a 6 carbon
9 carboxylic acid linker on the 5'-end. This product was synthesized using the
10 appropriate phosphoramidites on a standard synthesizer. The acid was then
11 reacted with 3-amino-1,2-propanediol in the presence of 1-(3-
12 dimethylaminopropyl)-3-ethylcarbodiimide to yield a stable diol. The diol was
13 oxidized to the aldehyde stage that was subsequently reacted with hydrazide
14 latex beads to form Schiff base linkages that were reduced with sodium
15 cyanoborohydride. The authors indicated that the oligonucleotide diol was a
16 stable intermediate but that the aldehyde should be prepared immediately
17 before coupling to the latex bead to minimize undesirable reaction of the
18 aldehyde with the oligonucleotide bases.

19 Another article of special interest as background to the present
20 invention is by *Tsarev et al.* (Biorg. Khim., 16: 765-79 (1990)) that describes
21 coupling of an aromatic aldehyde to the 5' phosphate of an oligonucleotide
22 through alkylation. The product was used to probe the enzyme-T7A2
23 promoter complex.

24 Typically, glass surfaces are activated by the introduction of amino-,
25 sulfhydryl-, carboxyl- or epoxyl- groups to the glass using the appropriate
26 siloxane reagent. Specifically, immobilization of oligonucleotide arrays on
27 glass supports has been described: by *Guo et al.*, Nuc. Acid Res., 22: 5456-
28 5465 (1994) using 1,4-phenylene diisothiocyanate; by *Joos et al.*, Anal.
29 Biochem., 247: 96-101 (1997) using succinic anhydride and carbodiimide

1 coupling; and by *Beatti, et al.*, Mol. Biotech., 4: 213-225 (1995) using 3-
2 glycidoxypolytrimethoxysilane.

3 The rapid specific reaction of cytidine in single stranded DNA with
4 semicarbazide moiety containing reagent, in the presence of bisulfite, has also
5 been described (*Hayatsu*, Biochem., 15: 2677-2682 (1976)).

6 Procedures which utilize arrays of immobilized oligonucleotides, such
7 as sequencing by hybridization and array-based analysis of gene expression
8 are known in the art. In these procedures, an ordered array of oligonucleotides
9 of different known sequences is used as a platform for hybridization to one or
10 more test polynucleotides, nucleic acids or nucleic acid populations.
11 Determination of the oligonucleotides which are hybridized and alignment of
12 their known sequences allows reconstruction of the sequence of the test
13 polynucleotide. See, for example, U.S. Patent Nos. 5,492,806; 5,525,464;
14 5,556,752; PCT Publications WO 92/10588, WO 96/17957 and the scientific
15 publications by *Ramsay*, Nat. Biotechnol., 16: 40-4 (1998) and by *Lipshutz et*
16 *al.*, Nat. Genet., 21: 20-24 (1999)).

17 However, many of the current immobilization methods suffer from one
18 or more of a number of disadvantages. Some of these are, complex and
19 expensive reaction schemes with low oligonucleotide loading yields, reactive
20 unstable intermediates prone to side reactions and unfavorable hybridization
21 kinetics of the immobilized oligonucleotide. The efficient immobilization of
22 oligonucleotides on glass surface in arrays in a high-through put mode requires
23 a) simple reliable reactions giving reproducible loading for different batches,
24 b) stable reaction intermediates, c) arrays with high loading and fast
25 hybridization rates, d) high temperature stability, e) low cost, and f) low
26 background.

27 The present invention represents a significant step in the direction of
28 meeting or approaching several of these objectives.

SUMMARY OF THE INVENTION

1
2 In accordance with the present invention a Schiff base type covalent
3 linkage is formed between a group containing an NH_2 moiety and an aromatic
4 aldehyde or ketone to covalently link an oligonucleotide (ODN) to a solid
5 support. The Schiff base type linkage is between the solid support and either
6 the 3', or 5' end of the ODN, or between the solid support and one or more
7 intermediate nucleotides in the ODN. Alternatively the Schiff base type
8 linkage is located in a combination of these sites. In this regard it should be
9 understood that the Schiff base type covalent linkage may be situated not
10 directly on the solid support or the ODN but on linking groups (linkers) which
11 are themselves covalently attached to the solid support and to the ODN,
12 respectively. Thus, either the solid support or the ODN or both may include a
13 linking group that includes the $-\text{NH}_2$ or aromatic aldehyde group which forms
14 the Schiff base type covalent bond to join the ODN to the solid support.

15 In accordance with one aspect and preferred mode or embodiment of
16 the invention the Schiff base type covalent bond is formed between a
17 semicarbazide group or moiety of the formula $\text{R}'\text{-NH-CO-NH-NH}_2$, and the
18 aromatic aldehyde moiety of the formula
19 $\text{R}''\text{-Q-CHO}$, preferably a benzaldehyde moiety, where the group R' designates
20 either the solid support or the ODN residue including any linker group
21 attached to the solid support or ODN, and where the R'' designates the other of
22 said solid support or ODN residues including any linker group attached to
23 them. The symbol Q in this formula designates an aromatic ring or a
24 heteroaromatic ring which may have up to three heteroatoms independently
25 selected from N, O and S, and where the aromatic or heteroaromatic ring may
26 itself be substituted with alkyl, alkoxy or halogen groups where the alkyl or
27 alkoxy group preferably has 1 to 6 carbons. The linkage formed between the
28 solid support and the ODN is thus depicted by the formula



1 where the symbols have the meaning provided above.

2 In accordance with still another aspect and preferred mode or
3 embodiment of the invention the semicarbazide moiety is attached to a glass
4 surface, and the benzaldehyde moiety is attached with a linker to the 3', or to
5 the 5' end of the ODN, or to one or more nucleotides situated internally in the
6 ODN. The synthetic methodologies to prepare the semicarbazide modified
7 solid support surface and the aromatic aldehyde coupled ODNs comprise still
8 further aspects of the present invention.

9 Advantages of the solid support ODN conjugates linked together with
10 the above-summarized Schiff base type linkages including an aromatic
11 aldehyde or ketone, and particularly with semicarbazone linkages, include (a)
12 their ability to be formed below pH 7, (b) stability of the Schiff base-with-
13 aromatic-aldehyde bonds and particularly of the semicarbazone-formed-with-
14 an-aromatic-aldehyde bonds, (c) ability to attach a high percentage (typically
15 more than 60 %, and preferably about 90 %, even more preferably 95 % or
16 more) of the ODN to the semicarbazide moiety containing solid support, and
17 (d) obtaining high coupling densities (preferably of about 10^4
18 oligonucleotides/ μm^2 and most preferably about 10^5 oligonucleotides/ μm^2) on
19 unit surface of the solid support. These advantages are to be contrasted with
20 the prior art procedures, see for example [*Kremsky et al.* (Nuc. Acid Res., 15:
21 2891-2909 (1987))] where an aliphatic aldehyde attached to the ODN is
22 coupled with a hydrazone-containing solid support to form a hydrazone that is
23 unstable and must be reduced to provide a stable solid support-ODN
24 conjugate.

25 Another aspect of the present invention is a general method for the
26 isolation of single stranded DNA in a process where an aldehyde-labeled
27 primer is used and an amplicon is immobilized on a semicarbazide containing
28

1 solid support. Denaturation of the amplicon and separation yield single
2 stranded DNA in solution and on the solid support, which could be used
3 individually for many applications known in the art. This is an improvement
4 and further development of the procedure described by *Mitchell et al.*, *Anal.*
5 *Biochem.*, 178: 239-42 (1989), where single-stranded DNA is "affinity
6 generated" following a polymerase chain reaction using a biotinylated primer,
7 followed by streptavidin-solid support separation.

8 In accordance with still another aspect or embodiment of the present
9 invention the oligonucleotides linked to the solid surface with the
10 semicarbazone bonds also contain one or more appropriately attached minor
11 groove binder moieties, a fluorescent generating moiety and a fluorescent
12 quencher. This conjugate is designed such that during amplification reactions,
13 with a perfect complementary target, the quencher molecules are cleaved
14 during amplification by the 5'-nuclease activity or a polymerase (as described
15 in United States Patent No. 5,210,015 and in *Witter et al.*, *Biotechniques* 22:
16 130-138 (1997)) resulting in a fluorescent immobilized oligonucleotide.
17 Mismatched targets are not amplified and no fluorescent signal is generated.
18 The specification of United States Patent No. 5,210,015 and the publication
19 *Witter et al.*, *Biotechniques* 22: 130-138 (1997) are expressly incorporated
20 herein by reference.

21 In accordance with yet another aspect of the present invention, non-
22 specific adsorption of the negatively charged nucleic acids to the
23 semicarbazone or other Schiff base-modified glass surface can be largely
24 eliminated by converting the unreacted NH_2 groups (preferably semicarbazide
25 $-\text{R}'\text{-NH-CO-NH-NH}_2$ groups) into a moiety containing an anion. This is
26 accomplished by reacting the solid support ODN conjugate with a reagent that
27 introduces an anionic group, for example by reacting the conjugate with 4-
28 formyl-1,3-benzenedisulfonate group. In addition, unreacted silanol functions

1 on the solid support, preferably glass surface are end-capped with a
2 hydrophobic siloxane to increase stability of the immobilized oligonucleotides.
3 Although this is not usually necessary, the semicarbazone linkages
4 formed with the aromatic aldehyde moiety and linking the oligonucleotide
5 with the solid support can be reduced to provide still stable solid-support-ODN
6 conjugates.

7 In accordance with a still further aspect of the present invention an
8 ODN containing cytidine is immobilized on a solid support containing
9 semicarbazide groups by bisulfite catalyzed covalent attachment through the
10 cytidine nucleotides of the ODN.

11 The present invention is primarily used at present for the capture and
12 detection of nucleic acids using oligonucleotides attached to glass surfaces
13 with the Schiff base type, (preferably semicarbazone) bonds, and more
14 particularly for the capture and detection of PCR generated nucleic acid
15 sequence in array format, although the use of the invention is not limited in
16 this manner. Generally speaking the oligonucleotides immobilized on solid
17 support in accordance with the present invention exhibit superior direct
18 capture ability for complementary oligonucleotide, DNA and RNA sequences.

19 BRIEF DESCRIPTION OF THE DRAWINGS

20 **Figure 1** is a graph showing in three dimensions the
21 optimization of oligonucleotide attachment via semicarbazone bonds to a glass
22 surface as a function of concentration of the oligonucleotide and pH of the
23 medium.

24 **Figure 2** is a graph showing the attachment of oligonucleotide to
25 the glass surface as expressed in units of fmol/spot as a function of time.

26 **Figure 3** is a graph showing the efficiency of hybridization and
27 efficiency of oligonucleotide attachment as a function of oligonucleotide
28 concentration applied on each spot.

1 **Figure 4** is the depiction of a hybridization of macroarray
2 consisting of six ODN probes to eight different 30-mer ODN targets, the
3 sequences of which are disclosed in Table 1, wherein each oligonucleotide is
4 spotted in triplicate giving an array of 3×6 spots and wherein the target
5 sequences 1 and 8 correspond to X and Y copy of the amelogenin gene and
6 wherein all other target sequences contain nucleotide substitutions at positions
7 indicated in bold in Table 1 and wherein match or mismatch of the base pairs
8 formed between each probe and the target are indicated at the bottom of each
9 ODN triplicate.

10 **Figure 5** is a depiction of a hybridization of the same
11 macroarray of six ODN probes shown in Figure 4 to single stranded 235-mer
12 PCR products generated from female or male human genomic DNAs and to
13 132-mer product representing isolated male copy of amelogenin gene
14 fragment, and wherein the PCR product generated from male DNA sample
15 represents a heterozygous equimolar mixture of female and male copies of the
16 gene fragment.

17 **Figure 6** is a schematic depiction of a solid support tethered 5'
18 nuclease assay.

19 DESCRIPTION OF THE PREFERRED EMBODIMENTS

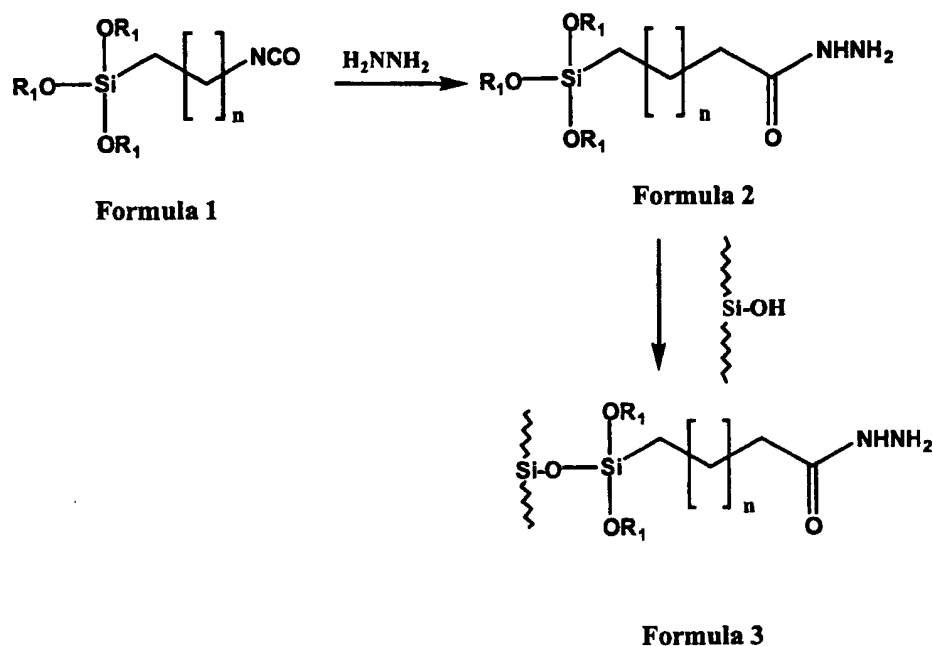
20 Derivatized Supports

21 As is noted in the Summary, in accordance with the present invention
22 one of the solid support or the oligonucleotide (ODN) contains a nucleophilic
23 amino group while the other contains an aromatic or heteroaromatic aldehyde
24 or ketone capable of reacting with the nucleophilic amino group to form a
25 Schiff base-type covalent linkage that attaches the ODN to the solid support,
26 in a reasonably fast, high yield reaction resulting in high concentrations of the
27 ODN per unit surface of the solid support, bound thereto by a stable covalent
28 bond. In order to have these properties the nucleophilic amino group

1 preferably and ideally has a pKa less than 7.0 In the preferred embodiments
2 the nucleophilic amino (NH_2) group is covalently linked to the solid support
3 while the aromatic aldehyde or ketone (preferably aldehyde) is linked to the
4 ODN.

5 Thus, the solid supports used in the preferred embodiments of
6 the invention contain the nucleophilic NH_2 group, as a primary amine (R' -
7 NH_2), or as a hydrazinyl,
8 ($\text{R}'\text{-NH-NH}_2$), oxyamino (O-NH_2), or semicarbazido ($\text{R}'\text{-NH-CO-}$
9 NH-NH_2) group. R' simply denotes the rest of the solid support, including a
10 possible linking group or linker). Most preferably the solid support in
11 accordance with the present invention includes a semicarbazido group attached
12 to the matrix of the solid support with a linker containing more than one atom
13 and less than 30 atoms. These amino (NH_2) group containing moieties can be
14 introduced on to the solid support or surface by methods known in the art.

15 Among the several types of solid supports available in the art
16 glass is most preferred. In accordance with this preferred embodiment of the
17 invention the glass surface contains the nucleophilic amino (NH_2) group,
18 which, as noted above, may be primary amino hydrazinyl, oxyamino, or a
19 semicarbazido group, linked to the glass surface with a linker containing more
20 than one atom and less than 30 atoms. Most preferably a semicarbazido group
21 is linked to the glass surface with the linker. The semicarbazido group has a
22 pKa less than 7.0. The semicarbazido and other amino (NH_2) groups can be
23 introduced on to the glass surface by methods known in the art, involving a
24 reaction with an appropriate trialkyloxysilane. For the most preferred
25 embodiments of the invention the semicarbazido group is introduced to the
26 glass surface with a semicarbazide containing trialkyloxysilane, as is shown in
27 **Reaction Scheme 1.**



Reaction Scheme 1

14

15

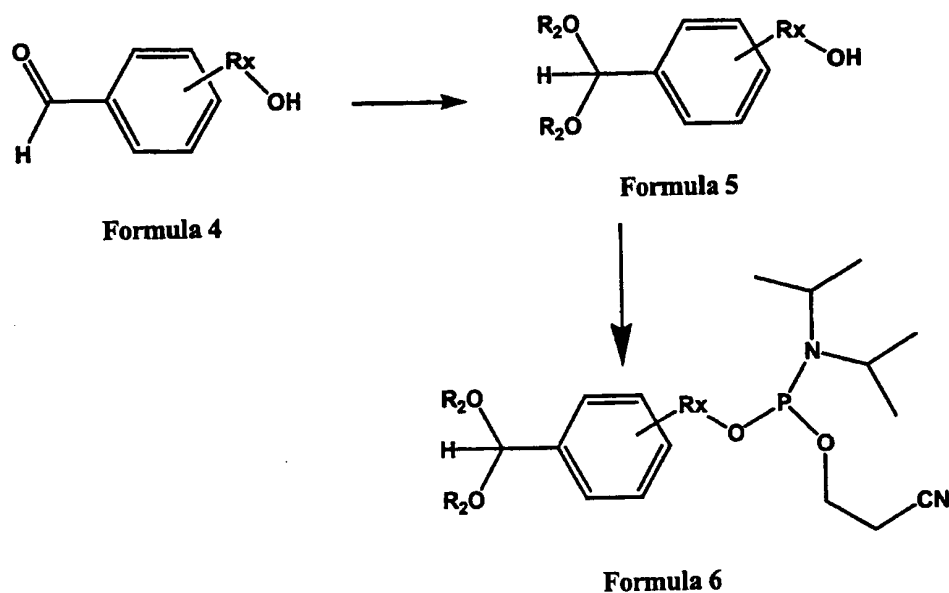
16 In Reaction Scheme 1 R_1 represents an alkyl group of 1 to 10 carbons,
 17 although one or more of the R_1 groups can also be phenyl. In the presently
 18 most preferred embodiment R_1 is ethyl. n is an integer, preferably having the
 19 values of 0 to 30, even more preferably 0 to 10. Thus, in accordance with this
 20 scheme a trialkoxy siloxane compound (**Formula 1**) having an isocyanate group
 21 attached by an alkyl chain is reacted with hydrazine to provide a
 22 trialkoxysilane including a semicarbazide (**Formula 2**), which is thereafter
 23 reacted with the glass surface to provide a glass surface (solid support) having
 24 a semicarbazide groups attached through the linker $(\text{CH}_2)_n$ (**Formula 3**). A
 25 detailed description of the conditions of these reactions is provided in the
 26 experimental section of this application for patent.

1 Derivatized Oligonucleotides

2 In the preferred embodiments of the invention an aromatic or
3 heteroaromatic aldehyde is covalently linked to the oligonucleotide (ODN), so
4 as to enable the ODN to react with the nucleophilic NH_2 (preferably
5 semicarbazide) groups linked to the solid support.

6 Prior to the current invention methods for the introduction of
7 aldehyde groups into oligonucleotides were complicated and required post
8 oligo-synthesis periodate oxidation of a diol precursor, as described by
9 *O'Shannessy et al.*, in *Anal. Biochem.*, 191: 1-8 (1990)). It is a novel aspect
10 or feature of the present invention to provide a phosphoramidite reagent that
11 includes a protected aromatic aldehyde and which can be used for the
12 introduction of the aldehyde group into an ODN during standard automated
13 oligonucleotide synthesis. In the most preferred embodiments the aromatic
14 aldehyde group or moiety is the "benzaldehyde" moiety having a linker
15 designated " R_x " attached to the phenyl ring, as illustrated in **Reaction**
16 **Scheme 2 by Formula 4.**

1

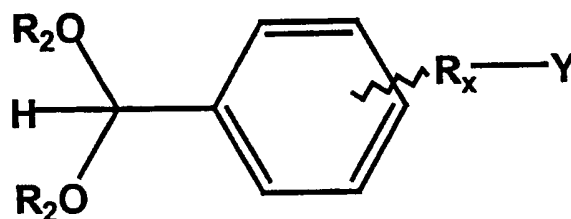


Reaction Scheme 2

2

3 In **Formula 4** the symbol R_x represents a chain of atoms, which may
 4 include a ring, and which may have the overall length of 2 to 150 atoms. R_x
 5 may contain atoms selected from C, H, N, O and S and in addition may
 6 contain one or more of $-NH-$, $-O-$, $-NH-C(=O)-$, $-C(=O)-NH-$, $-NH-$
 7 $C(=O)-NH-$, $-NH-C(=S)-NH-$, $-S-$, $OP(O)(O^-)O$ or $-S-S-$ groups. Synthetic
 8 methods to construct R_x are known in the art and are described, for example,
 9 in United States Patent No. 5,849,482 in connection with the description of
 10 synthesizing linker arms. The specification of United States Patent No.
 11 5,849,482 is expressly incorporated herein by reference. It should be
 12 understood that instead of the aromatic aldehyde of **Formula 4**, an aromatic
 13 ketone (such as acetophenone) could also be used, although the use of the
 14 aldehyde is preferred.

1 In accordance with **Reaction Scheme 2** the aromatic aldehyde (or
2 ketone) of **Formula 4** is protected in the aldehyde group by formation of a
3 diacetal, cyclic acetal or dialkanoate derivative of **Formula 5**. In **Formula 5**
4 R_2 represents an alkyl group of 1 to 6 carbons, an acyl group of one to 6
5 carbons, or the two R_2 groups together form a carbocyclic ring of 2 - 4 carbons
6 (as in a cyclic acetal, for example in a cyclic acetal formed with ethylene
7 glycol). The protected aldehyde of **Formula 5** is then converted into a
8 phosphoramidite reagent of **Formula 6**, as is shown in the reaction scheme.
9 Detailed experimental conditions for this conversion are described for an
10 example in the experimental section. The phosphoramidite reagent of
11 **Formula 6** is then used later to introduce the protected aldehyde into an
12 oligonucleotide (as described below.

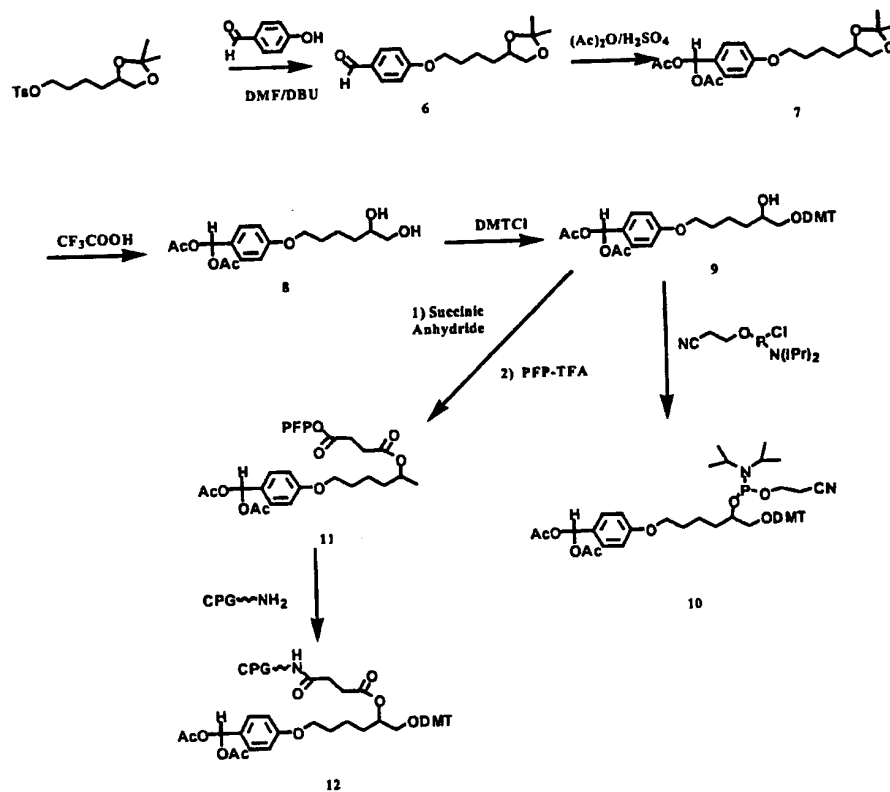


Formula 7

25 Instead of the phosphoramidite reagent of **Formula 6** the protected
26 aromatic aldehyde can also be attached to a primary or secondary amino group
27 that is itself attached to the 5' or to the 3' end of an ODN, or to a primary or
28 secondary amino group that is attached to an internal nucleotide in the ODN.

1 Amino-tailed ODNs can be prepared in accordance with the state-of-the-art,
2 and are described for example in United States Patent No. 5,512,667 the
3 specification of which is incorporated herein by reference. A reagent that is
4 suitable for attaching a protected aromatic aldehyde to said amino groups at
5 either tail end of the ODN or to one or more internal nucleotides is shown in
6 **Formula 7**. In **Formula 7** R_2 and R_x are defined as in connection with
7 **Formula 5**. Y is a reactive group (capable of reacting with a nucleophilic
8 amine), such as a carbonate, isocyanate, isothiocyanate, mono or di-substituted
9 pyridine, aziridine, CO-X, SO₂-X (X is halogen), monochlorotriazine,
10 dichlorotriazine, hydroxysulfosuccinimide ester, hydroxysuccinimide ester,
11 azodonitrophenyl or azido group. As example it is noted that an appropriately
12 activated 3-(α,α -dimethoxytolu-4-yl)propionic acid derivative can be coupled
13 to 5-(3-aminopropyl) uridine nucleotide incorporated in the ODN as an
14 internal base.

16



Reaction Scheme 3

1 **Reaction Scheme 3** discloses an actual example for the
2 synthesis of a controlled pore glass reagent **12** suitable for the synthesis of 3'-
3 aromatic-aldehyde-tailed oligonucleotides. In this regard it should be
4 understood that in the present description the numbers given to actual
5 compounds are to be distinguished from numbers given to general formulas.
6 Thus, the compound designated "**6**" in **Reaction Scheme 3** is to be
7 distinguished from **Formula 6** in **Reaction Scheme 2**. A detailed description
8 of the exemplary reaction conditions leading to the protected aldehyde
9 function attached to a controlled pore glass support designated **12** in the
10 scheme, is provided in the experimental section. The CPG support **12** includes
11 a dimethoxytriphenylmethyl (DMT) protecting group on a primary hydroxyl
12 function. After the DMT protecting group is removed an ODN can be built in
13 step-wise fashion on this support, in accordance with steps known in the art,
14 resulting in an ODN where the aromatic aldehyde moiety is attached to the 3'
15 end. The ODN, still having the aromatic aldehyde at its 3' end is then removed
16 from the solid support by methods well established in the state of the art.

17 **Reaction Scheme 3** also discloses an exemplary synthetic route
18 to provide a phosphoramidite reagent **10**, where the aldehyde function is
19 protected as the di-acetate. The phosphoramidite reagent **10** can be used in
20 accordance with the state of the art for synthesizing ODNs where the aromatic
21 aldehyde function is at the 5' end of the ODN.

22 The experimental section describes the conditions utilized for
23 purification and deprotection of the diacetal and diacetate derivatives used in
24 connection with this invention. The presence of an aldehyde group in the
25 oligonucleotide that was synthesized utilizing the aldehyde containing support
26 and/or the phosphoramidite reagent **10** can be confirmed with a reaction with
27 2,4-dinitrophenylhydrazine, followed by reversed phase HPLC analysis. This
28 technique clearly distinguishes the resulting hydrazone-ODN from starting

1 aldehyde -ODN. The aldehyde ODNs prepared in accordance with the
2 present invention showed no noticeable change in reactivity when stored at
3 -20°C for months.

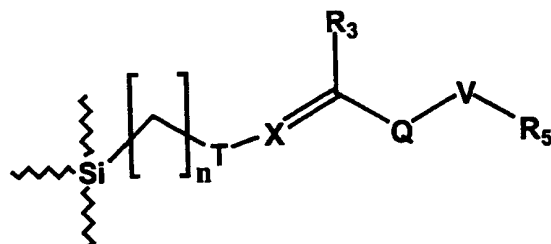
4 Coupling of the ODNs with the Modified Solid Support

5 The nucleophilic amino groups on the solid glass surface, as described
6 above for the preferred embodiment, are reacted with the aldehyde groups
7 attached to the 3'- or 5'- and of the ODN or to an internal base. Or
8 alternatively, as described briefly above, the aromatic aldehyde is attached to
9 the solid support (glass surface) and the amino group (preferably
10 semicarbazide) is attached to the ODN.

11 Generally the coupling reactions are performed at pH's between 2 and 7,
12 preferably at pH 6 and most preferably at pH 5. It has been found that, except
13 for the pH the reaction conditions are not critical for the reaction. It was
14 found, especially when semicarbazide NH_2 groups are used as in the preferred
15 embodiment, that high concentrations of ODN per unit surface of the glass
16 support can be achieved in accordance with the invention. Preferably
17 concentrations of 10^4 oligonucleotides/ μm^2 and more preferably 10^5
18 oligonucleotides/ μm^2 are obtained in accordance with the invention. The
19 semicarbazone linkage was determined to be stable at neutral and moderate
20 basic pH's used in standard PCR and diagnostic assays.

21 Moreover, as another aspect or feature of the present invention an
22 essentially background free solid support surface is achieved by treating the
23 un-reacted NH_2 groups on the solid support with an anionic generating
24 reagent.

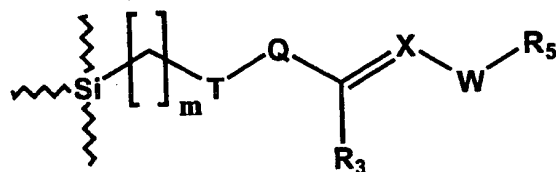
25 Exemplary glass-oligonucleotide conjugate products formed from reaction
26 of the nucleophilic amino group containing solid support with the aldehyde
27 derivitized oligonucleotide are shown in Formula 8



Formula 8

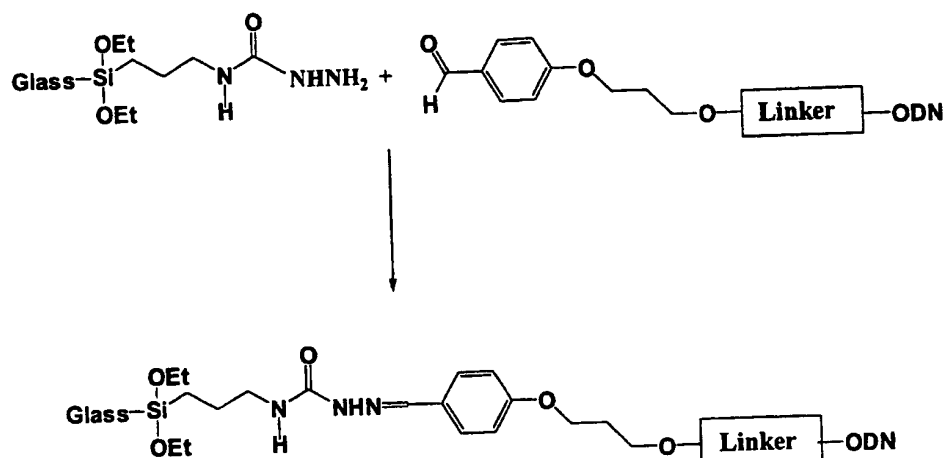
where n is 1 to 30; R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl; X is $-N=$; $-ON=$; $-C(=O)-NH-N=$; $-NH-C(=O)-NH-N=$ or $-NH-O-C(=O)-NH-N=$; Q is an aromatic ring which may be carbocyclic and may be a condensed ring structure such as naphthalene, dihydro or tetrahydronaphthalene, or a heteroaromatic ring that may be 5 or 6 membered (e. g. thiophene or pyridine) or a heteroaromatic ring that is part of a condensed ring structure, such as quinoline, and where the ring itself may be substituted with substituents such as lower alkyl, lower alkoxy or halogen); V is a linker that can be 2 to 100 atoms long and may contain atoms selected from C, H, N, O and S and in addition may contain one or more of $-NH-$, $-OH$, $-O-$, $-NH-C(=O)-$, $-C(=O)-NH-$, $-NH-C(=O)-NH-$, $-NH-C(=S)-NH-$, $-S-$, $OP(O)(O^-)O-$ or $-S-S-$ groups; R_5 is $-O-P(=O)(-U)-3'$ -oligomer of nucleotides or $-O-P(=O)(-U)-5'$ -oligomer of nucleotides where U is O or S. T represents a valence bond or a linker like V . T has a carbon atom adjacent to X .

An alternative exemplary preferred embodiment where an aldehyde modified solid support is coupled to an ODN containing a nucleophilic amino group at 3'-, 5'- or an internal base is shown by **Formula 9**.



Formula 9

where m is 1 to 30; R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl; X is $-N=$; $-ON=$; $-C(=O)-NH-N=$; $-NH-C(=O)-NH-N=$ or $-NH-O-C(=O)-NH-N=$; Q is an aromatic ring which may be carbocyclic and may be a condensed ring structure such as naphthalene, dihydro or tetrahydronaphthalene, or a heteroaromatic ring that may be 5 or 6 membered (*e. g.* thiophene or pyridine) or a heteroaromatic ring that is part of a condensed ring structure, such as quinoline, and where the ring itself may be substituted with substituents such as lower alkyl, lower alkoxy or halogen); W is 2 to 100 atoms long and may contain atoms selected from C, H, N, O and S and in addition may contain one or more of $-NH-$, $-OH$, $-O-$, $-NH-C(=O)-$, $-C(=O)-NH-$, $-NH-C(=O)-NH-$, $-NH-C(=S)-NH-$, $-S-$, $OP(O)(O^-)O-$ or $-S-S-$ groups; R_5 is $-O-P(=O)(-U)-3'$ -oligomer of nucleotides or $-O-P(=O)(-U)-5'$ -oligomer of nucleotides where U is O or S; T represents a valence bond or a linker like W . W and T have a carbon atom adjacent to X . Thus it should be understood that in **Formulas 8** and **9** the groups V , W and T represent the possible linker groups attaching the Schiff base type bond to the solid support and to the ODN, as applicable.



Reaction Scheme 4

1

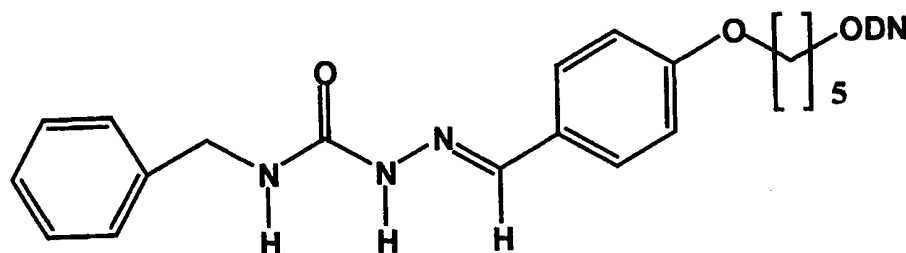
2 **Reaction Scheme 4** discloses the formation of an ODN--to-glass conjugate
 3 linked with semicarbazone bonds in accordance with the presently most
 4 preferred embodiment of the invention.

5 In still other embodiments, the oligonucleotide is attached to the solid
 6 support through more than one type of aromatic aldehyde containing moiety
 7 introduced at either the 3', 5' or at internal nucleotides.

8 It is also within the scope of the present invention to immobilize a long
 9 chain DNA to a solid support that contains a semicarbazide moiety as
 10 disclosed above. with bisulfite catalyzed covalent attachment through cytidine
 11 residues, in analogy to the reaction described by *Hayatsu* in *Biochem.*, 15:
 12 2677-2682 (1976), incorporated herein by reference.

13 Stability of the semicarbazone linkage under PCR assay conditions were
 14 determined using a model compound shown below:

15



Formula 10

The semicarbazone conjugate (Formula 10) was treated in a PCR buffer at 95°C for 30 minutes and analyzed by reversed phase HPLC chromatography. Comparison of the treated semicarbazone conjugate with the starting material showed little or no degradation.

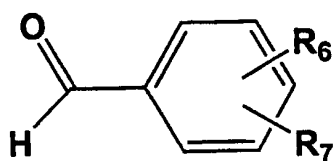
In another embodiment a solid surface linked oligonucleotides also contain appropriately held, minor groove binder, fluorescent generating moiety and a fluorescent quencher. This conjugate is designed such that during amplification reactions with a perfect complementary target the quencher molecules are cleaved during amplification by the 5'-nuclease activity, in analogy to the reaction described in United States Patent No. 5,210,015 and in *Witter al.*, *Biotechniques* 22: 130-138 (1997), resulting in a fluorescent immobilized oligonucleotide. Mismatched targets are not amplified and no fluorescent signal is generated. This is schematically in Figure 6. The chemistries and methods to attach a the minor groove binder (MGB), fluorophore (F) and quencher (Q) to an ODN has been described in US 5,801,155, and in co-pending application serial number 09/054,832, filed on April 3, 1998, the specifications of which are incorporated herein by reference.

Materials for construction of arrays include, but are not limited to, nylon,

1 polypropylene, nitrocellulose, glass, silicon wafers, optical fibers, copolymers and
2 other materials suitable for construction of arrays such as are known to those of
3 skill in the art.

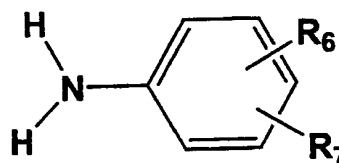
4 Endcapping of unreacted groups on the solid surface

5 After the covalent attachment of the oligonucleotide the solid support via the
6 semicarbazone linkage, the unreacted amino groups on the surface are treated with
7 anion generating reagents aiding to limit non-specific primer and amplicon
8 background. This is achieved by treatment of the solid surface with appropriate
9 aromatic aldehydes (**Formula 11**). Similarly, when a semicarbazide-labeled
10 oligonucleotide is coupled to aromatic aldehyde containing solid support, the
11 unreacted aldehyde groups are reacted with anion generating reagents (**Formula**
12 **12**), where R_6 and R_7 are independently H-, $-COO^-$ or $-SO_3^-$. Unreacted silanol
13 groups can also be modified to further enhance surface characteristics. The
14 appropriate silanes are commercially available (UCT, Bristol, PA).



20

21 **Formula 11**



22

23 **Formula 12**

24 Hybridization Characteristics of Modified Solid Supports

25 The oligonucleotide loadings on the solid surface were determined by the
26 use of 5'-or 3'-aldehyde-modified oligonucleotides ^{32}P -labeled at opposing ends
27 using the appropriate nucleotide triphosphate ^{32}P -labeled and either terminal
28 deoxynucleotidyl transferase or T4 polynucleotide kinase. The ^{32}P -labeled
oligonucleotide was reacted directly with the semicarbazide modified glass surface

1 Oligonucleotide Arrays

2 In another embodiment of the present invention, immobilized oligonucleotides
3 are used in procedures which utilize arrays of oligonucleotides, such as sequencing
4 by hybridization and array-based analysis of gene expression. In sequencing by
5 hybridization, an ordered array of oligonucleotides of different known sequences is
6 used as a platform for hybridization to one or more test polynucleotides, nucleic
7 acids or nucleic acid populations. Determination of the oligonucleotides which are
8 hybridized and alignment of their known sequences allows reconstruction of the
9 sequence of the test polynucleotide. Alternatively, oligonucleotides comprising the
10 wild-type sequence and all possible mutant sequences for a given region of a gene
11 of interest can be placed on an array. Exposure of the array to DNA or RNA from a
12 subject or biological specimen, under hybridization conditions, allows
13 determination of wild-type or mutant status for the gene of interest. This is
14 described, without using the present invention, in the prior art, for example in U.S.
15 Patent Nos. 5,492,806; 5,525,464; 5,556,752; PCT publications WO 92/10588
16 and WO 96/17957, all of which are incorporated herein by reference. Both of the
17 foregoing techniques require discrimination between related sequences, especially
18 at the single-nucleotide level; hence, the simplicity, reproducibility of solid support
19 attachment oligonucleotides of the invention provides improvements in these
20 techniques. Materials for construction of arrays include, but are not limited to,
21 nylon, polypropylene, nitrocellulose, glass, silicon wafers, optical fibers,
22 copolymers and other materials suitable for construction of arrays such as are
23 known to those of skill in the art.

24 An additional application of the present invention to array technology is in the
25 examination of patterns of gene expression in a particular cell or tissue. In this
26 situation oligonucleotides or polynucleotides corresponding to different genes are
27 arrayed on a surface, and a nucleic acid sample from a particular cell or tissue type,
28 for example, is incubated with the array under hybridization conditions. Detection
29 of the sites on the array at which hybridization occurs allows one to determine

1 which oligonucleotides have hybridized, and hence which genes are active in the
2 particular cell or tissue from which the sample was derived.

3 Array methods can also be used for identification of mutations, where wild-type
4 and mutant sequences are placed in an ordered array on a surface. Hybridization of
5 a polynucleotide sample to the array under stringent conditions, and determination
6 of which oligonucleotides in the array hybridize to the polynucleotide, allows
7 determination of whether the polynucleotide possesses the wild-type or the mutant
8 sequence. Since many mutant sequences of clinically-relevant genes differ from
9 their wild-type counterpart at only one or a few nucleotide positions, the enhanced
10 discriminatory powers of the modified oligonucleotides of the invention provides
11 improvements in mutation detection. Array methods can also be used in any
12 diagnostic procedure where nucleic acid hybridization is feasible in combination
13 with an appropriate detection system. The nucleic acids include DNA, RNA and
14 sequences amplified by methods known in the art.

15 In all of the above-mentioned applications of array technology, the simplicity
16 and efficiency of oligonucleotide attachment to solid supports in accordance with
17 the invention provides significant improvements in manufacturing and performance
18 of the arrays.

19 General

20 The availability of oligonucleotides containing an aldehyde linker directly from
21 the oligonucleotide synthesizer allows the immobilization of oligonucleotides to
22 any amine containing solid support. Thus oligonucleotide affinity chromatography
23 material can be readily synthesized in accordance with the invention. In addition,
24 the use of a primer labeled at the 3'-end with an aldehyde allows facile
25 immobilization of the amplicon, after amplification, to an amine containing solid
26 surface and allows the isolation of single strands after denaturation.

27 EXAMPLES

28 The following examples are included for illustrative purposes only and are not

1 intended to limit the scope of the invention.

2 General Experimental

3 All air and water sensitive reactions were carried out under a slight positive
4 pressure of argon. Anhydrous solvents were obtained from Aldrich (Milwaukee,
5 WI). Flash chromatography was performed on 230-400 mesh silica gel. Melting
6 points were determined on a Mel-Temp melting point, apparatus in open capillary
7 and are uncorrected. Elemental analysis was performed by Quantitative
8 Technologies Inc. (Boundbrook, NJ). UV-visible absorption spectra were
9 recorded in the 200-400-nm range on a UV-2100 (Shimadzu) or a Lambda 2
10 (Perkin Elmer) spectrophotometers. ¹H NMR spectra were run at 20°C on a
11 Bruker WP-200 or on a Varian XL-200 spectrophotometer; chemical shifts are
12 reported in ppm downfield from Me₄Si. Thin-layer chromatography was run on
13 silica gel 60 F-254 (EM Reagents) aluminum-backed plates.

14 Example 1. Preparation of (a,a-Dimethoxytolu-4-yl)-oxyethyl, 2-cyanoethyl N,N-
15 diisopropylphosphoramidite (5).

16 4-Hydroxyethoxybenzaldehyde dimethyl acetal (3)

17 To a solution of 4-hydroxyethoxybenzaldehyde (*Bernstein et al.*, J. Am. Chem.
18 Soc., 73: 906-912 (1951); 8.5 g, 51.2 mmol), 2,2-dimethoxypropane (30 mL, 244
19 mmol) in a mixture of methanol (40 mL) and CH₂Cl₂ (100 mL) was added
20 anhydrous Amberlyst 15 (Aldrich) (1.0 g). The mixture was stirred for 5 hrs, the
21 catalyst was removed by filtration and the filtrate was concentrated to give the
22 crude product contaminated with the starting aldehyde. This material was
23 chromatographed on silica eluting with 1:1 ethyl acetate-hexane. The pure product
24 fractions were pooled and concentrated. Drying under vacuum afforded 7.6 g (70
25 %) of the title compound as a pale yellow, viscous liquid. ¹H NMR: δ 7.28 (d, J= 9
26 Hz, 2 H), 6.92 (d, J=9 Hz, 2H), 5.31 (s, 1H), 4.86 (t, J=5.5 Hz, 1H), 3.98 (t, J=5
27 Hz, 2H), 3.71 (q, J=5 Hz, 2H), 3.20 (s, 3H). ¹³C NMR: δ 158.60, 130.19, 127.74,
28 113.90, 102.46, 69.43, 59.52, 52.27.

29 (a,a-Dimethoxytolu-4-yl)-oxyethyl, 2-cyanoethyl N,N-diisopropylphosphoramidite

1 (5).

2 To a solution of 1 (4.76 g, 22.45 mmol) and ethyldiisopropylamine (10 mL) in
3 50 mL of anhydrous CH_2Cl_2 was added 2-cyanoethyl
4 diisopropylchlorophosphoramidite (5.85 g, 24.7 mmol). After being stirred for 1 h,
5 the reaction was treated with methanol (1 mL) to quench excess phosphitylating
6 agent and diluted with CH_2Cl_2 . The solution was washed with 5% sodium
7 bicarbonate, brine and dried over Na_2SO_4 . Concentration under vacuum gave an oil
8 which was chromatographed on silica eluting with hexane-ethyl acetate-
9 triethylamine (2:1:0.1). The desired product was obtained as a colorless, viscous
10 syrup (6.3 g, 68 %) after solvent evaporation and drying in *vacuo*.

11 Example 2. Preparation of Acetyloxy[4-(6-[bis(4-
12 methoxyphenyl)phenylmethoxy]-5-[[bis(methylethyl)amino]-(2-
13 cyanoethoxy)phosphinoxy}hexyloxy)phenyl]methyl acetate (10)
14 4-[4-(2,2-Dimethyl-1,3-dioxolan-4-yl)butoxy]benzaldehyde (6).

15 A solution of 4-hydroxybenzaldehyde (2.83 g, 23.22 mmol), toluene-4-sulfonic
16 acid 4-(2,2-dimethyl-1,3-dioxolan-4-yl)-butyl ester (*Lehmann et al.*,
17 *Carbohydr. Res.*, 169: 53-68 (1987); 7.62 g, 23.22 mmol) and 1,8-
18 diazabicyclo[5,4.0]undec-7ene (3.6 ml) in 50 ml of anhydrous DMF was stirred at
19 85 °C for 4h. The DMF was removed in *vacuo* and the residue was purified by
20 silica gel chromatography eluting with 30% ethyl acetate in hexane. The pure
21 product fractions were evaporated affording a homogenous oil: 4.93 g (75%)
22 yield; TLC (1:1, ethyl acetate/hexane), R_f = 0.68; ^1H NMR (CDCl_3) 9.89 (1H, s,
23 aldehyde), 7.83 (2H, d, J = 8.9 Hz, aromatic), 6.98 (2H, d, J = 8.8 Hz, aromatic),
24 (2H, m, CH_2), 4.06 (2H, t, J = 6.6 Hz, CH_2), 3.53 (1H, t, J = 7.1 Hz, CH), 1.86 (2H,
25 m, CH_2), 1.60 (2H, m, CH_2), 1.41 and 1.36 (6H, 2 x s, methyl). Anal. Calcd for
26 $\text{C}_{16}\text{H}_{22}\text{O}_4 \cdot 0.15 \text{H}_2\text{O}$: C, 68.38; H, 8.00. Found: C, 68.31; H, 8.08.

27 Acetyloxy{4-[4-(2,2-dimethyl(1,3-dioxolan-4-yl))butoxy]phenyl}methyl acetate
28 (7).

29 Sulfuric acid (1.0 ml of a 1% solution in acetic anhydride) was added to a

1 solution of **3** (4.78 g, 17.13 mmol) in 60 ml of acetic anhydride. The solution
2 was stirred for 90 min at room temperature and then poured into 500 ml of ice-cold
3 5% sodium bicarbonate solution. The product was extracted into ethyl acetate
4 (500 ml) and the extract was washed with water (2 x 500 ml), dried over sodium
5 sulfate and evaporated affording **7** as an oil: 5.89 g (90%) yield; TLC (1:1, ethyl
6 acetate/hexane), $R_f = 0.73$; $^1\text{H NMR}$ (CDCl_3) 7.62 (1H, s, acetal CH), 7.43 (2H,
7 d, $J = 8.7$ Hz, aromatic), 6.90 (2H, d, $J = 8.6$ Hz, aromatic), 4.08 (2H, m, CH_2),
8 3.97 (2H, t, $J = 6.5$ Hz, CH_2), 3.52 (1H, t, $J = 7.1$ Hz, CH), 2.11 (6H, s, acetyl),
9 1.82 (2H, m, CH_2), 1.60 (2H, m CH_2), 1.41 and 1.36 (6H, 2 x s, methyl). Anal.
10 Calcd for $\text{C}_{20}\text{H}_{28}\text{O}_7$: C, 63.14; H, 7.42. Found: C, 63.19; H, 7.40.

11 Acetyloxy[4-(5,6-dihydroxyhexyloxy)phenyl]methyl acetate (**8**).

12 Trifluoroacetic acid (1.5 ml) was added to a solution of **7** (5.8 g, 15.26 mmol)
13 in 20% aqueous methanol. The solution was stirred for 40 min at room
14 temperature and then diluted with 400 ml of ethyl acetate and washed with 400 ml
15 of 5% sodium bicarbonate solution followed by 400 ml of water. The organic
16 solution was dried over sodium sulfate and evaporated. The residue was purified
17 by silica gel chromatography eluting with a gradient of 50% hexane in ethyl
18 acetate to 100% ethyl acetate to 5% methanol in ethyl acetate. The pure product
19 fractions were evaporated affording an oil: 1.1 g (20%) yield; TLC (5% methanol
20 in ethyl acetate), $R_f = 0.64$; $^1\text{H NMR}$ (CDCl_3) 7.62 (1H, s, acetal CH), 7.44 (2H,
21 d, $J = 8.7$ Hz, aromatic), 6.90 (2H, d, $J = 8.6$ Hz, aromatic), 3.98 (2H, t, $J = 6.3$ Hz,
22 CH_2), 3.68 (1H, m, CH), 3.46 (2H, m, CH_2), 2.11 (6H, s, Acetyl), 1.82 (2H, m,
23 CH_2), 1.55 (4H, m, CH_2). Anal. Calcd for $\text{C}_{17}\text{H}_{24}\text{O}_7$: C, 59.99; H, 7.11. Found:
24 C, 60.26; H, 7.08.

25 Acetyloxy(4-{6-[bis(4-methoxyphenyl)phenylmethoxy]-5-hydroxyhexyloxy}-
26 phenyl)methyl acetate (**9**).

27 Dimethoxytrityl chloride (1.21 g, 3.57 mmol) was added to a solution of **8** (1.0
28 g, 2.94 mmol) in 17 ml of dry pyridine. The solution was stirred at room
29 temperature for 2 h. and then poured into 250 ml of 5% sodium bicarbonate and

1 extracted with 300 ml of ethyl acetate. The extract was dried over sodium sulfate
2 and evaporated. The residue was purified by silica gel chromatography eluting
3 with 50% hexane in ethyl acetate (1% triethylamine). The pure product fractions
4 were pooled and evaporated affording a foam: 1.66 g (85%) yield; TLC (1:1,
5 ethyl acetate/hexane), $R_f = 0.50$; ^1H NMR (CDCl_3) 7.62 (1H, s, acetal CH), 7.43
6 (2H, d, $J = 8.2$ Hz, aromatic), 7.33 - 6.81 (17H, aromatic), 3.92 (2H, t, $J = 6.4$ Hz,
7 CH_2), 3.78 (6H, s, OCH_3), 3.17 (1H, dd, $J = 3.3$ and 9.4 Hz, CH), 3.03 (1H, t, $J =$
8 7.7 Hz, CH), 2.37 (1H, m, CH), 2.10 (6H, s, acetyl), 1.81 - 1.38 (6H, multiplets,
9 CH_2). Anal. Calcd for $\text{C}_{38}\text{H}_{42}\text{O}_9$: C, 71.01; H, 6.59. Found: C, 70.91; H, 6.42.
10 Acetyloxy[4-(6-[bis(4-methoxyphenyl)phenylmethoxy]-5-
11 {[bis(methylethyl)amino]-(2-cyanoethoxy)phosphinoxy}hexyloxy)phenyl]methyl
12 acetate (10)

13 2-Cyanoethyl diisopropylchlorophosphoramidite (0.49 ml, 2.19 mmol) was
14 added to a solution of 9 (0.83 g, 1.29 mmol) dissolved in 32 ml of anhydrous
15 methylene chloride containing 0.67 ml of *N,N*-diisopropylethylamine. The
16 reaction solution was stirred for 1.0 h at 25°C under argon and then treated with
17 1.0 ml of methanol and poured into 300 ml of 5% sodium bicarbonate solution.
18 The mixture was extracted with ethyl acetate (300 ml) and the extract was dried
19 over sodium sulfate and evaporated. The crude product was purified by silica gel
20 chromatography eluting with a gradient of 25-50% ethyl acetate in hexane (2%
21 triethylamine). The pure phosphoramidite fractions were evaporated affording a
22 homogenous oil: 0.61 g (56%) yield; TLC (1:1, ethyl acetate/hexane), $R_f = 0.62$;
23 ^{31}P NMR ($\text{DMSO}-d_6$) 147.82 (singlet). Anal. Calcd for $\text{C}_{47}\text{H}_{59}\text{N}_2\text{O}_{10}\text{P} \cdot 0.2 \text{H}_2\text{O}$:
24 C, 66.68; H, 7.07; N, 3.31. Found: C, 66.46; H, 7.27; N, 2.94.

25 Example 3. Preparation of CPG (12)

26 A solution of 9 (0.83 g, 1.29 mmol), succinic anhydride (0.15 g, 150 mmol),
27 triethylamine (0.2 ml) and *N*-methyl imidazole (12 μl) in 3.0 ml of dry methylene
28 chloride was stirred at room temperature under argon for 14 h. Pentafluorophenyl
29 trifluoroacetate (0.39 ml, 2.32 mmol) was added and the solution was stirred for an

1 additional 30 min. The reaction solution was loaded onto a silica gel column and
2 eluted with 25% ethyl acetate in hexane (0.5% triethylamine included). The pure
3 product fractions were pooled and evaporated affording a surup: 467-mg (40%)
4 yield; TLC (1:1, ethyl acetate/hexane), R_f = 0.56; ^1H NMR (CDCl_3) 7.62 (1H, s,
5 acetal CH), 7.42 (2H, d, J = 8.3 Hz, aromatic), 7.33 - 6.72 (17H, aromatic), 5.16
6 (1H, t, J = 5.8 Hz, CH), 3.90 (2H, t, J = 6.0 Hz, CH_2), 3.77 (6H, s, methoxys), 3.16
7 (2H, m, CH_2), 3.01 (2H, t, J = 6.4 Hz, succinyl CH_2), 2.80 (2H, t, J = 6.5 Hz,
8 succinyl CH_2), 2.11 (6H, s, acetyls), 1.82 - 1.32 (6H, multiplets, CH_2). Anal. Calcd
9 for $\text{C}_{48}\text{H}_{45}\text{F}_5\text{O}_{12}$: C, 63.43; H, 4.99. Found: C, 63.65; H, 4.71.

10 Attachment of 11 to CPG (12)

11 To a suspension of controlled pore glass (LCAA 500 A,
12 4.2 g; loading, 283 $\mu\text{mol/g}$) in 13.0 ml of anhydrous DMF was added 11 (226 mg,
13 0.252 mmol) and triethylamine (1.5 ml). The mixture was gently shaken under argon
14 for 24 h. Anhydrous pyridine (84 ml) was then added followed by acetic anhydride
15 (84 ml). The mixture was shaken for 1.0 h. The beads were filtered, rinsed with
16 DMF and methanol and dried: loading- 41 $\mu\text{mol/g}$.

17 Example 4 Preparation of 3-(4-Semicarbazido)propyltriethoxysilane

18 Anhydrous hydrazine (3.2 ml; Aldrich, Milwaukee, WI) were dissolved in
19 30 ml of anhydrous acetonitrile. 2.5 g of isocyanatopropyltriethoxysilane (United
20 Chemical Technologies, Bristol, PA) were added dropwise with vigorous stirring.
21 Reaction mixture was stirred for 1 h at room temperature and the solvent was
22 removed in vacuum. Oily residue was dissolved in anhydrous ethanol, the solution
23 was filtered, and the solvent and unreacted hydrazine were evaporated under
24 reduced pressure. The last step was repeated twice omitting filtration. The
25 resulting viscous residue was dried in vacuum overnight to afford 2.7 g (yield
26 96%) of the desired product as a clear oil. ^1H NMR: d 6.82 (s, 1H, NH), 6.32 (t,
27 J =5.4 Hz, 1H, NH), 4.05 (br s, 2H, NH_2), 3.72 (q, J =7 Hz, 6H, CH_2), 2.96 (q, J =6.6
28 Hz, 2H, CH_2), 1.41 (m, 2H, CH_2), 1.26 (t, J =6.9 Hz, 9H, CH_3), 0.49 (m, 2H, CH_2).

1 Example 5 Oligonucleotide Synthesis

2 All oligonucleotides were synthesized on an ABI 392 DNA/RNA
3 synthesizer using standard phosphoramidite chemistry. The oligonucleotides were
4 purified by reverse-phase HPLC, and their concentrations determined by UV
5 spectrophotometry at 260 nm (Ref) Yield was similar to that observed in normal
6 oligonucleotide synthesis.

7 Example 6 Derivatization of Glass Slides and Preparation of Oligonucleotide

8 Arrays

9 Preparation of Slides

10 Glass slides were derivatized according to the standard silanization procedure
11 described below. Pre-cleaned microscope slides (Corning Glass Works, Corning,
12 NY) were treated with 1N HNO₃ for 1 h at room temperature and then rinsed with
13 running deionized water followed by anhydrous ethanol wash. The slides were
14 then immersed in 1% 3-(4-semicarbazido)propyltriethoxysilane solution in 95%
15 ethanol/water for 30 min. The slides were washed with 95% ethanol for 5 min,
16 twice with acetonitrile, 5 min per wash, and finally with ether. After that the slides
17 were cured for 45 min at 110°C. The derivatized slides can be stored at least for a
18 month on a bench top in a dust proof container without noticeable loss of activity.

19 Immobilization of Oligonucleotides

20 Benzaldehyde-modified oligonucleotides were dissolved in 100 mM sodium
21 acetate buffer (pH 5.0) at the desirable concentration and spotted manually directly
22 on the derivatized slide as a 0.5 l droplets following a grid pattern on a wet paper
23 template underneath the slide. Slides were incubated at 37°C in a covered Petri
24 dish located in a humid container for 1-5 hours. To block all unreacted
25 semicarbazide groups on the glass surface the slides were treated with 100mM
26 solution of 4-formyl-1,3-benzenedisulfonic acid disodium salt in 100 mM sodium
27 acetate buffer (pH 5.0) for 1 h at 37°C. The slides were then rinsed with water,
28 washed for 30 min at 37°C with 30% methanol in 0.5 M sodium phosphate buffer
29 (pH 7.0) followed by a 30 min wash in 5' SSPE, 0.1% Triton X-100 at the same

1 temperature. The slides were rinsed thoroughly with water, air dried at room
2 temperature and were ready for use in hybridization experiments.

3 **Figure 1** shows the effect of different pH's and oligonucleotide concentration
4 on immobilization efficiency. A pH of 5 and an oligonucleotide concentration of
5 20 mM showed optimum immobilization on glass surfaces.

6 **Figure 2** shows that optimum oligonucleotide immobilization is achieved on
7 the glass surface in about 1 hour.

8 **Example 7** Determination of Oligonucleotide Loading and Hybridization 9 Efficiency

10 The 5' or 3' aldehyde-modified oligonucleotides were radioactively labeled
11 at the opposite end using [α - 32 P]ddATP (NEN, Boston, MD) and terminal
12 deoxynucleotidyl transferase (Promega, Madison, WI), or [γ - 32 P]ATP (Amersham,
13 Arlington Heights, IL) and T4 polynucleotide kinase (NE Biolabs, Beverly, MA),
14 respectively. Briefly, 1.2 nmol of oligonucleotide and 100 mCi of appropriate
15 radioactive triphosphate were taken into a labeling reaction using the conditions
16 specified by the manufacture. The labeled oligonucleotide was purified using
17 NENSORBä 20 cartridge (NEN, Boston, MD). Eluate from the cartridge
18 containing labeled oligonucleotide was dried down, dissolved in 100 μ l of 100
19 mM sodium acetate buffer (pH 5.0) and supplemented with 9 nmol of unlabeled
20 oligonucleotide to approximately 100 mM final concentration. Serial dilution of
21 this stock was made using the same buffer with a two fold decrement. 0.5 μ l of
22 oligonucleotide solutions at various concentrations were applied in quadruplicates
23 to semicarbazide-derivatized glass slide and allowed to react at 37°C for 3 h. The
24 glass surface was blocked and washed as described above, and bound
25 oligonucleotide was quantified by phosphor imaging using a Bio-Rad GS-250
26 Molecular Imager. The data from the phosphor imager were converted to fmol/spot
27 by comparing to standard curves generated from a serial dilution of known
28 amounts of the same labeled oligonucleotide probes spotted on a microscope slide
29 and dried down without any washing.

1 To determine hybridization efficiency or availability of attached
2 oligonucleotides for hybridization with a complementary target, an aldehyde-
3 modified non-radiolabeled probe was immobilized on a slide as described above.
4 2.4'5.0 cm cover slip was positioned over the area where the probes were spotted
5 using 0.2 mm thick spacers made from electric tape. 80-100 ml of hybridization
6 mixture (1 mM 5' ³²P-labeled complementary oligonucleotide, 5' SSPE, 0.1%
7 Triton X-100) was applied by capillary action between the slide and the cover slip.
8 The slide was incubated overnight at 37°C in a closed Petri dish over wet
9 Whatman 3MM paper in a humid container to prevent evaporation of the
10 hybridization solution. Two 30 min washes were performed on a shaker with 25 ml
11 per slide of hybridization buffer. The level of hybridization was quantified as
12 described above.

13 **Figure 3** investigates the effect of oligonucleotide concentration applied in
14 the immobilization reaction on the glass surface on covalent attachment and
15 hybridization efficiencies. As shown, optimum hybridization target capture starts
16 to occur at oligonucleotide applied concentrations of about 10 mM, that yields
17 covalently attached oligonucleotide of >200 fmol/2mm spot. Optimum
18 oligonucleotide target capture of about 75-100 fmol/2 mm spot occurs.

19 **Example 8 Hybridization of Oligonucleotide Arrays with Short**
20 **Oligonucleotide Targets or Single-stranded PCR Products**

21 Female and male human genomic DNA samples were obtained from Coriell
22 Institute of Medical Research (NIGMS Human Genetic Mutant Cell Repository,
23 Camden, NJ). The 235 bp amelogenine gene fragment corresponding to exon 3
24 was amplified by PCR using a set of primers, 5'-
25 GCTGCACCACCAAATCATCCC-3' (SEQUENCE ID No. 15) and 5'-biotin-
26 CTGGTGAGGCTGTGGCTGAAC-3' (SEQUENCE ID No. 16). The
27 amplification reaction (100 ml) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3),
28 1.5 mM MgCl₂, 0.001% gelatin, 100 ng DNA, 1 mM of each primer, 200 mM each
29 dATP, dCTP, dTTP and dGTP, and 2.5 units of JumpStart[®]Taq DNA polymerase

1 (Sigma, St. Louis, MO). PCR was performed in a Statagene RoboCycler Gradient
2 40 Temperature Cycler (Stratagene, La Jolla, CA) using 35 cycles (95°C for 1 min,
3 65°C for 1 min, 72°C for 1min).

4 The PCR products were purified by 4% non-denaturing polyacrilamide gel
5 electrophoresis. One DNA strand of PCR products derived from the non-
6 biotinylated primer was 5' end labeled using [γ -32P]ATP and T4 polynucleotide
7 kinase. This labeled strand was separated using streptavidin-coupled magnetic
8 beads Dynabeads M-280 (Dynal, Inc., Lake Success, NY) according to
9 manufacture's instructions. Briefly, 50 ml of labeling reaction mixture containing
10 1-2 mg of PCR product was diluted twice with 2'B&W buffer (10 mM Tris-HCl
11 (pH 7.5), 1 mM EDTA, 2 M NaCl) and added to 1 mg of pre-washed Dynabeads.
12 The suspension was incubated at room temperature for 15 min with occasional
13 mixing. Beads were separated using the magnet, washed three times with 100 ml
14 of B&W buffer and treated with 75 ml of 0.1 N NaOH to denature the DNA
15 strands. The mixture was incubated at room temperature for 5 min, supernatant
16 was collected, and denaturation step was repeated one more time. Combined
17 supernatants were neutralized with equal volume of 0.1 N HCl and ethanol
18 precipitated. The specificity of amplification was confirmed by sequencing the
19 labeled strand using Maxam and Gilbert procedure (Maxam, A.M., & Gilbert, W.
20 Proc. Natl. Acad. Sci. U.S.A. 79, 560-564(1977)).

21 Hybridization of oligonucleotide macroarrays consisting of six
22 oligonucleotides spotted in triplicates with 5' labeled 30-mer complementary
23 synthetic oligonucleotide targets was accomplished the same way as it had been
24 described in the previous section, except for the hybridization time, which was
25 reduced to 3 h. After that slides were washed for 15 min at room temperature in
26 hybridization buffer (5'SSPE, 0.1% Triton X-100). Slides were then washed 2'15
27 min at 42°C in 0.5'SSPE, 0.1% Triton X-100. In some cases an additional 15 min
28 wash was necessary to improve the mismatch discrimination. Finally, the slides
29 were air dried and analyzed by phosphorimaging.

1 To hybridize single stranded PCR product to the same array of
2 oligonucleotides, the concentration of the target was 10-20 nM. Overnight
3 hybridization at 37°C was followed by 15 min wash in hybridization buffer, and
4 2'15 min wash at 37°C in 0.5'SSPE, 0.1% Triton X-100.

5 The oligonucleotide sequences of the primers and probes used, are shown
6 in Table 1. The specificity of the capture using an array of capture oligonucleotides
7 is shown in **Figures 4 and 5**. Specifically, **Figure 4** is the depiction of a
8 hybridization of macroarray consisting of six ODN probes to eight different 30-
9 mer ODN targets, the sequences of which are disclosed in **Table 1**, wherein each
10 oligonucleotide is spotted in triplicate giving an array of 3×6 spots and wherein the
11 target sequences 1 and 8 correspond to X and Y copy of the amelogenin gene and
12 wherein all other target sequences contain nucleotide substitutions at positions
13 indicated in bold in **Table 1** and wherein match or mismatch of the base pairs
14 formed between each probe and the target are indicated at the bottom of each ODN
15 triplicate. **Figure 5** is a depiction of a hybridization of the same macroarray of six
16 ODN probes shown in **Figure 4** to single stranded 235-mer PCR products
17 generated from female or male human genomic DNAs and to 132-mer product
18 representing isolated male copy of amelogenin gene fragment, and wherein the
19 PCR product generated from male DNA sample represents a heterozygous
20 equimolar mixture of female and male copies of the gene fragment.

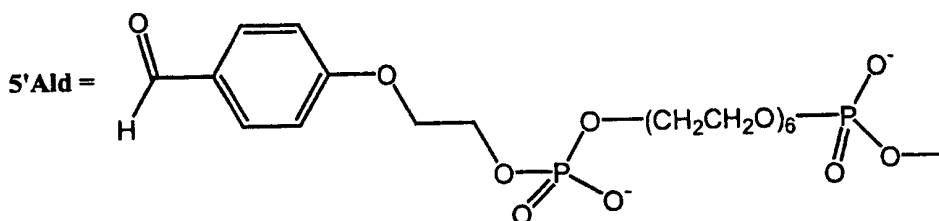
21 These results illustrate the reproducibility of the immobilization reactions.
22 In addition the hybridization results show the expected results for the indicated
23 match and mismatches.

24
25
26
27
28
29
30
31

Table 1. Sequences of Oligonucleotide Targets and Probes

ODN name and SEQUENCE ID No.	Sequence	Type of mismatch (probe name in parenthesis)
target 1	3'CAAACCGACCACCACAACCTAACCTCAGTACCTCAC 5'	C-T(2); T-G(4); C-A(6)
target 2	3'CAAACCGACCACCACAACCTAACCTCAGTACCTAAC 5'	A-G(1); T-G(4); C-A(6)
target 3	3'CAAACCGACCACCACAACCCAACCTCAGTACCTCAC 5'	C-T(2); C-A(3); C-A(6)
target 4	3'CAAACCGACTACCACAACCTAACCTCAGTACCTCAC 5'	C-T(2); T-G(4); T-G(4)
target 5	3'CAAACCGACCACCACAACCCAACCTCAGTACCTAAC 5'	A-G(1); C-A(3); C-A(6)
target 6	3'CAAACCGACTACCACAACCCAACCTCAGTACCTCAC 5'	C-T(2); C-A(3); T-G(5)
target 7	3'CAAACCGACTACCACAACCTAACCTCAGTACCTAAC 5'	A-G(1); T-G(4); T-G(5)
target 8	3'CAAACCGACTACCACAACCCAACCTCAGTACCTAAC 5'	A-G(1); C-A(3); T-G(5)
probe 1 SEQ. ID No. 9	5'Ald-TGGAGTCATGGAGTG 3'	
probe 2 SEQ. ID No. 10	5'Ald-TGGAGTCATGGATTG 3'	
probe 3 SEQ. ID No. 11	5'Ald-GTGTGGATTGGAGT 3'	
probe 4 SEQ. ID No. 12	5'Ald-GTGTGGGTTGGAGT 3'	
probe 5 ID No. 13	5'Ald-TTTGGCTGGTGGTG 3'	
probe 6 ID No. 14	5'Ald-TTTGGCTGATGGTG 3'	

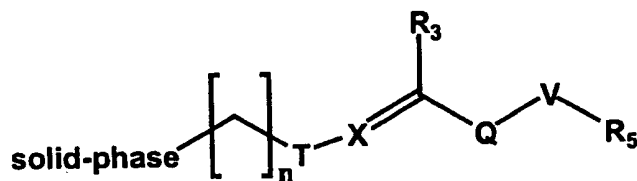
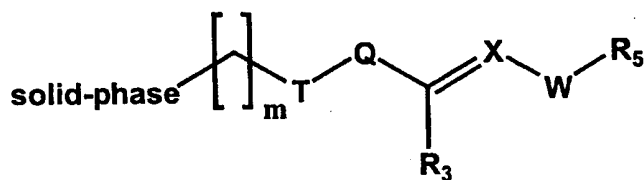
¹Targets 1 through 8 represent ODNs having SEQUENCE ID Nos. 1 - 8 assigned to them, respectively.



Formula 13

WHAT IS CLAIMED IS:

- 1
2 1. A composition of matter comprising an oligonucleotide attached to a
3 solid support, having the formula selected from the group consisting of **formula**
4 **(i)** and **formula (ii)**,

**formula (i)****formula (ii)**

22 where the symbol solid-phase represents a solid matrix to which the rest of
23 the composition is covalently attached;

24 $n=1$ to 30;

25 m is 1 to 30;

26 R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl;

27 X is $-\text{N}=\text{}$; $-\text{ON}=\text{}$; $-\text{C}(\text{O})-\text{NH}-\text{N}=\text{}$; $-\text{NH}-\text{C}(\text{O})-\text{NH}-\text{N}=\text{}$ or $-\text{NH}-\text{O}-\text{C}(\text{O})-$

28 $\text{NH}-\text{N}=\text{}$; Q is carbocyclic condensed or not-condensed aromatic ring, or a

29 condensed or not-condensed heteroaromatic ring, said carbocyclic or

1 heteroaromatic ring being optionally substituted with a lower alkyl, lower alkoxy
2 or halogen group;

3 V is a linker having the length of 2 to 100 atoms that contains carbon to
4 carbon bonds and optionally and independently may contain carbon to oxygen
5 bonds and one or more moieties selected from the group consisting of -NH-, -
6 OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-,
7 OP(O)(O⁻)O- and -S-S-;

8 W is a linker having the length of 2 to 100 atoms that contains carbon to
9 carbon bonds and optionally and independently may contain carbon to oxygen
10 bonds and one or more moieties selected from the group consisting of -NH-, -
11 OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-,
12 OP(O)(O⁻)O- and -S-S-, said linker W terminating with a carbon atom adjacent to
13 X;

14 T is a valence bond or a linker having the length of 1 to 100 atoms, that
15 contains carbon to carbon bonds and optionally and independently may carbon to
16 oxygen bonds and one or more moieties selected from the group consisting of -
17 NH-, -OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-
18 and -S-S-, said linker T terminating with a carbon atom adjacent to X.
19 R₅ is -O-P(=O)(-U)-3'-oligomer of nucleotides or -O-P(=O)(-U)-5'-oligomer of
20 nucleotides where U is O or S.

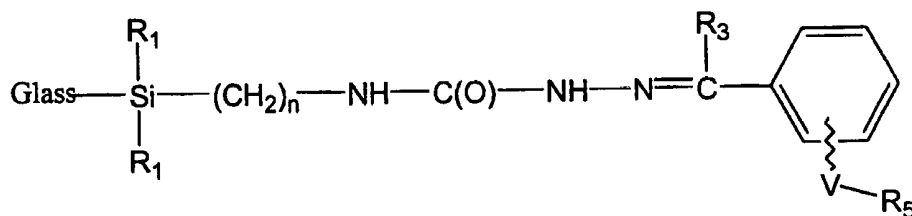
21 2. A composition of matter in accordance with Claim 1 in accordance with
22 formula (i).

23 3. A composition of matter in accordance with Claim 2 where X is -N= or
24 -NH-C(=O)-NH-N=.

25 4. A composition of matter in accordance with Claim 3 where X is
26 -NH-C(=O)-NH-N=.

27 5. A composition of matter in accordance with Claim 2 where Q represents
28 a benzene ring.

- 1 6. A composition of matter in accordance with Claim 4 where Q
2 represents a benzene ring.
- 3 7. A composition of matter in accordance with Claim 2 where the solid
4 phase is a glass surface.
- 5 8. A composition of matter in accordance with Claim 1 in accordance with
6 formula (ii).
- 7 9. A composition of matter in accordance with Claim 8 where X is -N= or
8 -NH-C(=O)-NH-N=.
- 9 10. A composition of matter in accordance with Claim 9 where X is
10 -NH-C(=O)-NH-N=.
- 11 11. A composition of matter in accordance with Claim 8 where Q
12 represents a benzene ring.
- 13 12. A composition of matter in accordance with Claim 8 where the solid
14 phase is a glass surface.
- 15 13. A composition of matter in accordance with Claim 2 where NH₂ groups
16 not attached to the oligonucleotide are end-capped with a covalently linked moiety
17 containing an anion.
- 18 14. A composition of matter comprising an oligonucleotide attached to a
19 glass support, having the formula



- 26 where Glass represents the glass support;
- 27 R₁ is alkyl of 1 to 10 carbons or phenyl;
- 28 n is an integer having the values 1 to 30;
- 29 R₃ is H or an alkyl group of 1 to 6 carbons;

1 **V** is a linker having the length of 2 to 100 atoms, that contains carbon to
2 carbon bonds and optionally and independently may contain carbon to oxygen
3 bonds and one or more moieties selected from the group consisting of -NH-, -
4 OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-,
5 OP(O)(O⁻)O- and -S-S-, and

6 **R₃** is -O-P(=O)(-U)-3'-oligomer of nucleotides or -O-P(=O)(-U)-5'-
7 oligomer of nucleotides where **U** is O or S.

8 **15.** A composition of matter in accordance with Claim 14 where **n** is 3.

9 **16.** A composition of matter in accordance with Claim 14 where **R₃** is H.

10 **17.** A composition of matter in accordance with Claim 14 where the phenyl
11 group is attached through linker **V** to the 5'-end of the oligonucleotide.

12 **18.** A composition of matter in accordance with Claim 14 where the phenyl
13 group is attached through linker **V** to the 3'-end of the oligonucleotide.

14 **19.** A composition of matter in accordance with Claim 14 where **n** is 3, **R₃**
15 is H, and the phenyl group is attached through linker **V** to the 5'-end of the
16 oligonucleotide.

17 **20.** A composition of matter in accordance with Claim 19 where the linker
18 **V** includes an OP(O)(O⁻)O- moiety.

19 **21.** A method of attaching an oligonucleotide to a solid support, to provide
20 a solid-support oligonucleotide conjugate, the method comprising the step of:

21 reacting a derivatized solid support of the **formula (iii)** with a derivatized
22 oligonucleotide of the **formula (iv)**, or by reacting a derivatized solid support of
23 the **formula (v)** with a derivatized oligonucleotide of the **formula (vi)**

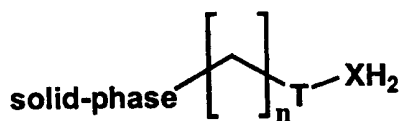
24

25

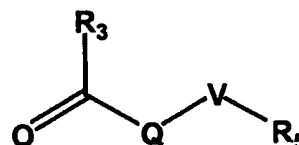
26

27

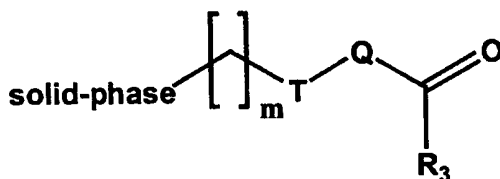
28



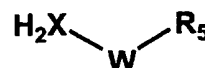
formula (iii)



formula (iv)



formula (v)



formula (vi)

where the symbol solid-phase represents a solid matrix;

$n=1$ to 30 ;

m is 1 to 30 ;

R_3 is H , $\text{C}_1\text{-C}_6\text{alkyl}$ or $\text{C}_3\text{-C}_6\text{cycloalkyl}$;

X is $-\text{N}$; $-\text{ON}$; $-\text{C}(=\text{O})-\text{NH}-\text{N}$; $-\text{NH}-\text{C}(=\text{O})-\text{NH}-\text{N}$ or $-\text{NH}-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{N}$;

Q is carbocyclic condensed or not-condensed aromatic ring, or a condensed or not-condensed heteroaromatic ring said carbocyclic or heteroaromatic ring being optionally substituted with a lower alkyl, lower alkoxy or halogen group;

V is a linker having the length of 2 to 100 atoms, that contains carbon to carbon bonds and optionally and independently may contain carbon to oxygen bonds and one or more moieties selected from the group consisting of $-\text{NH}-$, $-\text{OH}$, $-\text{O}-$, $-\text{NH}-\text{C}(=\text{O})-$, $-\text{C}(=\text{O})-\text{NH}-$, $-\text{NH}-\text{C}(=\text{O})-\text{NH}-$, $-\text{NH}-\text{C}(=\text{S})-\text{NH}-$, $-\text{S}-$, $\text{OP}(\text{O})(\text{O})\text{O}-$ and $-\text{S}-\text{S}-$;

W is a linker having the length of 2 to 100 atoms, that contains carbon to

1 carbon bonds and optionally and independently may contain carbon to oxygen
2 bonds and one or more moieties selected from the group consisting of -NH-, -
3 OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-,
4 OP(O)(O⁻)O- and -S-S-, said linker W terminating with a carbon atom adjacent to
5 X;

6 T is a valence bond or a linker having the length of 1 to 100 atoms, that
7 contains carbon to carbon bonds and optionally and independently may
8 CONTAIN carbon to oxygen bonds and one or more moieties selected from the
9 group consisting of -NH-, -OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-
10 NH-, -NH-C(=S)-NH-, -S- and -S-S-, said linker T terminating with a carbon atom
11 adjacent to X.

12 R₅ is -O-P(=O)(-U)-3'-oligomer of nucleotides or -O-P(=O)(-U)-5'-
13 oligomer of nucleotides where U is O or S.

14 22. A method in accordance with Claim 21 where a derivatized solid
15 support of the **formula (iii)** is reacted with a derivatized oligonucleotide of the
16 **formula (iv)**.

17 23. A method in accordance with Claim 22 where the reaction is conducted
18 in an aqueous phase having a pH less than approximately 8.

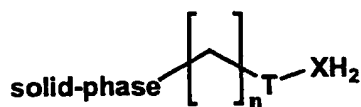
19 24. A method in accordance with Claim 23 where the reaction is conducted
20 in an aqueous phase having a pH less than approximately 7.

21 25. A method in accordance with Claim 22 where X is selected from the
22 group consisting of -N and -NH-O-C(=O)-NH-N.

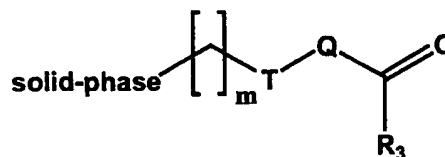
23 26. A method in accordance with Claim 22 R₃ is H and Q represents a
24 benzene ring.

25 27. A method in accordance with Claim 21 where a derivatized solid
26 support of the **formula (v)** is reacted with a derivatized oligonucleotide of the
27 **formula (vi)**.

28 28. A derivatized solid support of **formula (iii)** or of **formula (v)**



formula (iii)



formula (v)

where the symbol solid-phase represents a solid matrix;

$n=1$ to 30;

m is 1 to 30;

R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl;

X is -N; -ON; -C(=O)-NH-N; -NH-C(=O)-NH-N or -NH-O-C(=O)-NH-N;

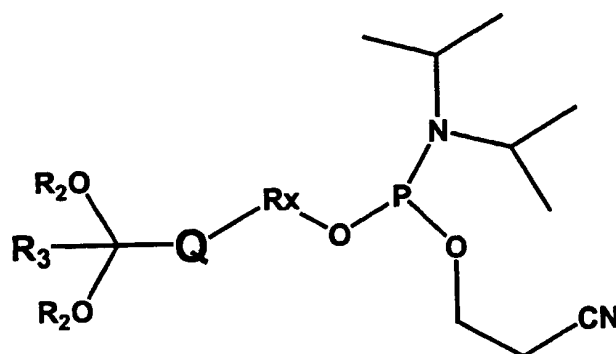
Q is carbocyclic condensed or not-condensed aromatic ring, or a condensed or not-condensed heteroaromatic ring said carbocyclic or heteroaromatic ring being optionally substituted with a lower alkyl, lower alkoxy or halogen group, and

T is a valence bond or a linker having the length of 1 to 100 atoms, that contains carbon to carbon bonds and optionally and independently may include carbon to oxygen bonds and one or more moieties selected from the group consisting of -NH-, -OH, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S- and -S-S-, said linker T terminating with a carbon atom adjacent to X .

29. A derivatized solid support in accordance with Claim 28 that is in accordance with formula (iii).

30. A derivatized solid support in accordance with Claim 29 where X is selected from the group consisting of -N and -NH-O-C(=O)-NH-N.

31. A phosphoramidite reagent of the formula



where R_2 is an alkyl group of 1 to 6 carbons, an acyl group of one to 6 carbons, or the two R_2 groups together form a carbocyclic ring of 2 - 4 carbons;

R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl;

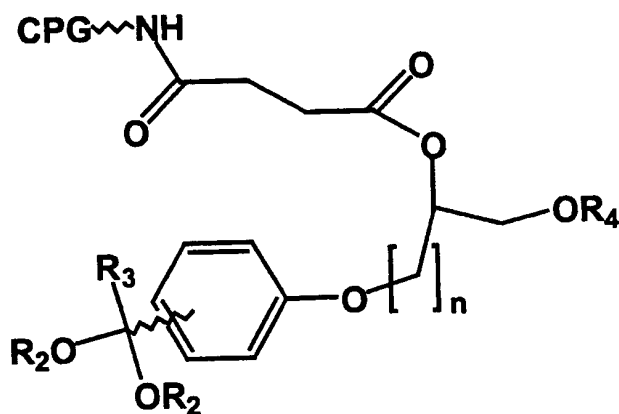
R_x is a chain of atoms, optionally including a ring, of an overall length of 2 to 150 atoms, including carbon to carbon bonds and optionally and independently including carbon to oxygen bonds and one or more moieties selected from the group consisting of -NH-, -O-, -NH-C(=O)-, -C(=O)-NH-, -NH-C(=O)-NH-, -NH-C(=S)-NH-, -S-, OP(O)(O⁻)O or -S-S- groups, and

Q is carbocyclic condensed or not-condensed aromatic ring, or a condensed or not-condensed heteroaromatic ring said carbocyclic or heteroaromatic ring being optionally substituted with a lower alkyl, lower alkoxy or halogen group.

32. A phosphoramidite reagent in accordance with Claim 31 where R_3 is H.

33. A phosphoramidite reagent in accordance with Claim 32 where Q represents a benzene ring.

34. A derivatized controlled pore glass support of the formula



where n is an integer having the values of 1 to 30 R_2 is an alkyl group of 1 to 6 carbons, an acyl group of one to 6 carbons, or the two R_2 groups together form a carbocyclic ring of 2 - 4 carbons;

R_3 is H, C_1 - C_6 alkyl or C_3 - C_6 cycloalkyl, and

R_4 is H or is dimethoxytriphenylmethyl.

35. A derivatized controlled pore glass support in accordance with Claim 34 where R_3 is H.

36. A derivatized controlled pore glass support in accordance with Claim 35 where n is 4 and R_2 is CH_3CO -.

37. A method of coupling a solid support to an oligonucleotide, the support having a matrix and $NH-C(O)-NHNH_2$ groups covalently attached to the matrix through a covalently attached linker group, the oligonucleotide including cytosine nucleosides, the method comprising the step of:

reacting in an aqueous phase in the presence of bisulfite the solid support with the oligonucleotide.

38. A method of binding a nucleic acid or a fragment thereof to a substantially complementary strand of oligonucleotide, said method comprising the steps of:

providing an array of oligonucleotides of varying sequences on a solid

- 1 support surface where each ODN is bound to the surface by a covalent bond
2 including a Schiff base formed between an NH_2 group and an aromatic aldehyde,
3 and
4 contacting the nucleic acid or fragment thereof with the array of
5 oligonucleotides bound to the solid surface.
- 6 39. The method of Claim 38 where the Schiff base is formed between a
7 semicarbazide group attached to the solid surface and an aromatic aldehyde
8 attached to each of the oligonucleotides.
- 9 40. The method of Claim 39 where the solid surface is a glass surface.

1/6

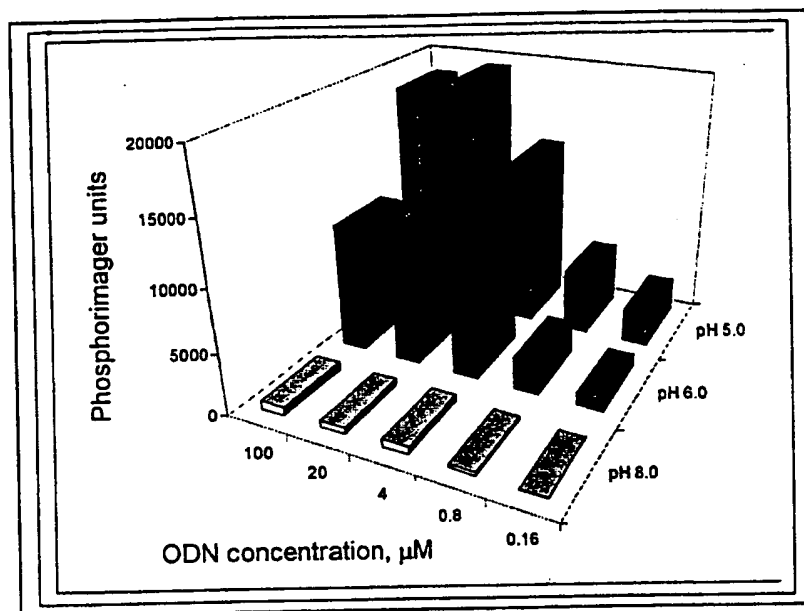


Fig. 1

2/6

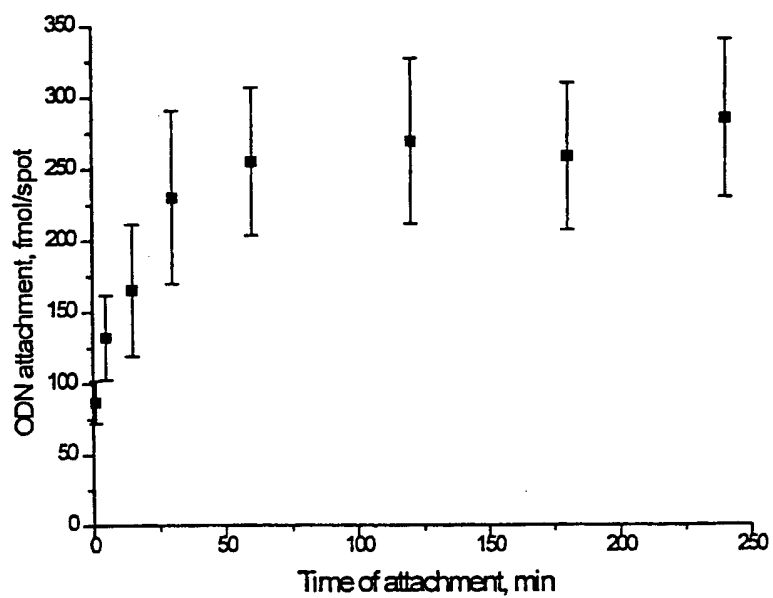


Fig. 2

3/6

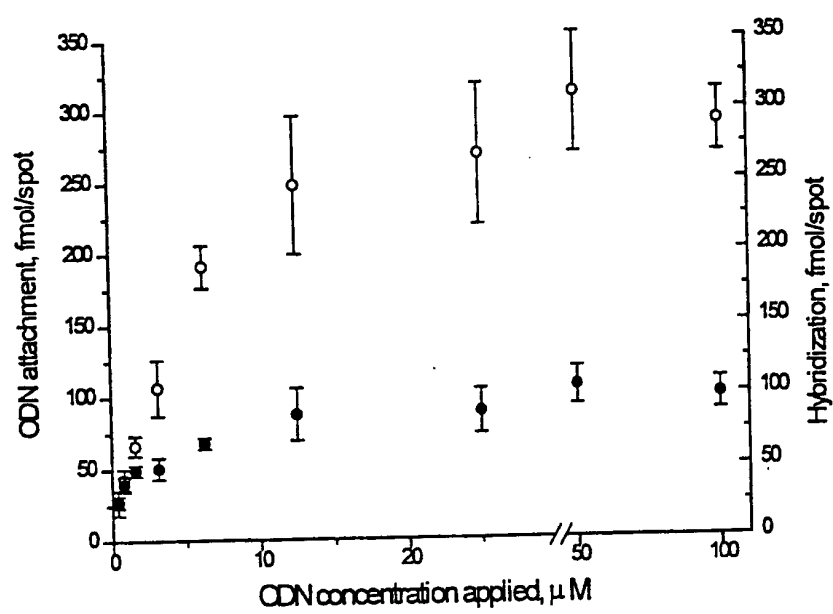


Fig. 3

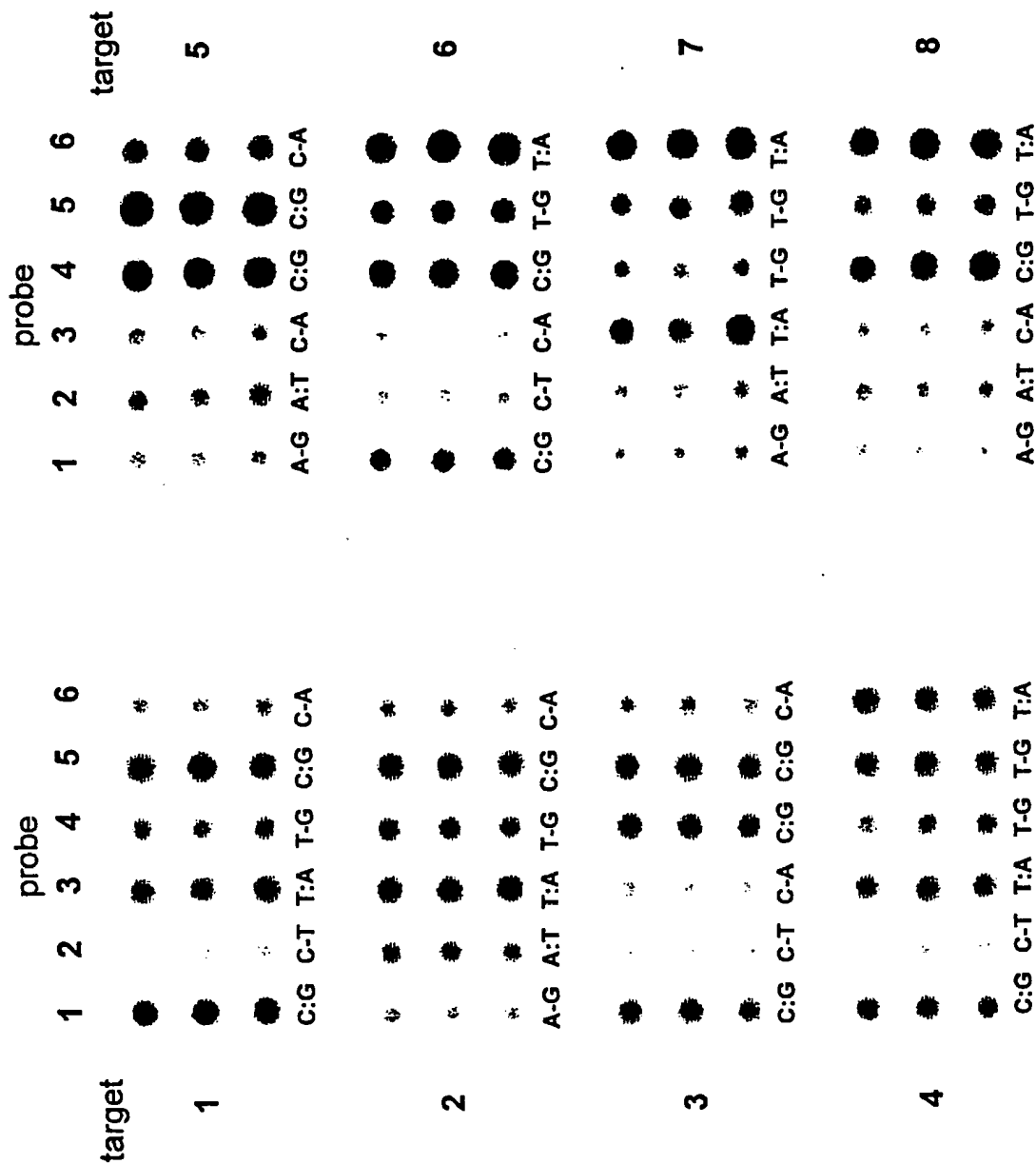


Fig. 4

5/6

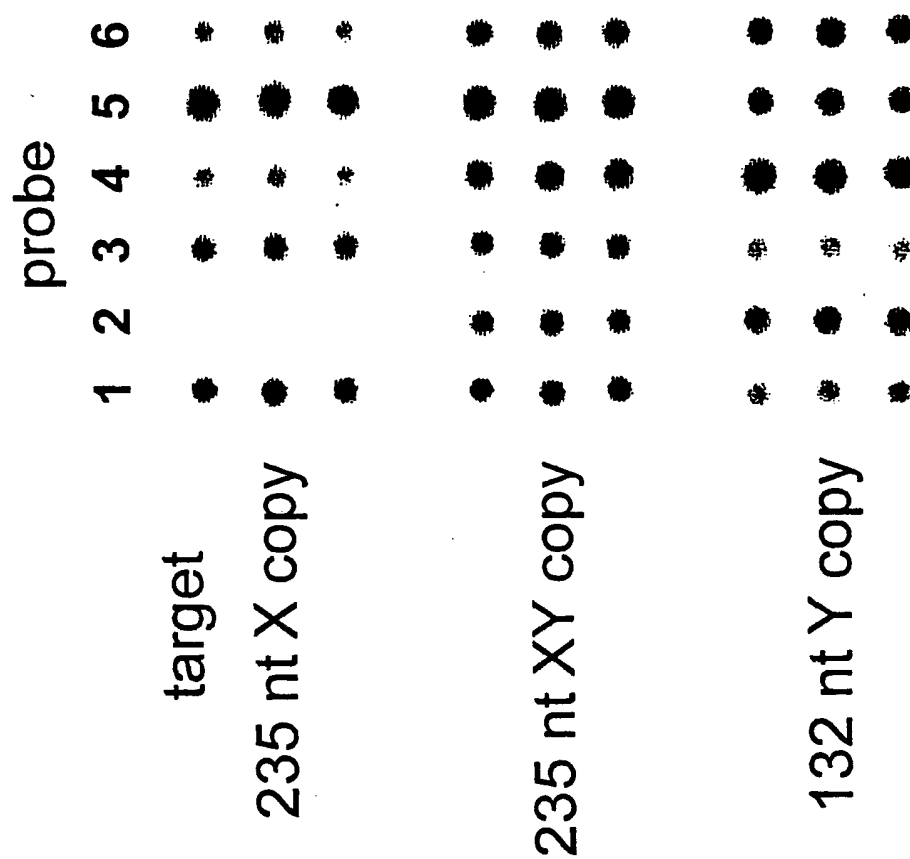


Fig. 5

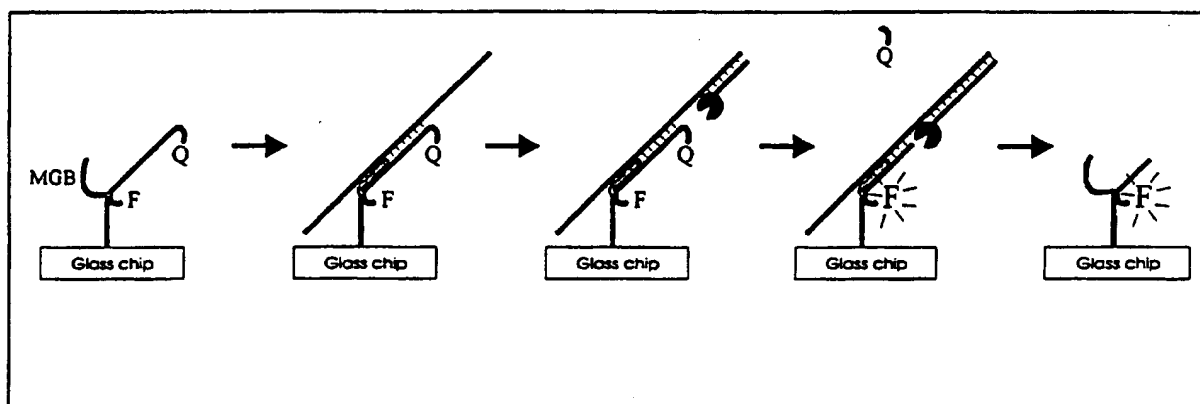


Fig. 6